An Investigation into the Nature of Hyperpolarizing Outward Current Enhanced by Pinacidil or Metabolic Inhibition in Isolated Smooth Muscle Myocytes.

by

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B.Sc.

A Thesis
Submitted to the Faculty of Graduate Studies
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ABSTRACT

K^+ channel opener drugs and metabolic inhibitors are believed to produce smooth muscle relaxation by causing hyperpolarization of membrane potential. However, the identity of the K^+ channel(s) which produce the hyperpolarization remains controversial. In this study, the properties of macroscopic and unitary K^+ currents found in isolated smooth muscle cells of the rabbit portal vein that were affected by the K^+ channel opener, pinacidil, or the metabolic inhibitors cyanide, 2,4-dinitrophenol (DNP) and 2-deoxy-D-glucose (2DG) were determined. Whole-cell current and single channel activity from cell-attached patches were measured via patch-clamp technique before and after exposure to pinacidil (50 - 100 μM), or a combination of cyanide (2 mM) or DNP (50 μM) and 2DG (10 μM). Pinacidil caused a simultaneous increase in; a) time-dependent outward current evoked during step voltage changes from -60 mV to between -80 and +30 mV, as well as, b) quasi steady-state outward current evoked between -95 and +10 mV during 8 second ramp protocols. Both these components were voltage-dependent, sensitive to intracellular Ca^{2+} chelation with 5 mM EGTA, inhibited by TEA^+ (0.5 mM) or charybdotoxin (20 nM) and insensitive to 4-aminopyridine (10 mM). Three different single channel conductances of approximately 50, 100, and 250 pS, were observed in on-cell patches of myocytes bathed in high K^+ (140 mM) solution (symmetrical K^+ recording condition). However, only the 250 pS channel demonstrated a reversible increase in open probability (NP_o increased from 0.012 to 0.025) by the presence of pinacidil. Metabolic inhibition by cyanide + 2DG caused a rapid increase in a voltage-dependent outwardly rectifying

steady-state current as well as an outward shift in holding current. The onset of this current caused a shift in the reversal potential of the whole-cell steady-state I-V relation towards E_K. Inclusion of 5 mM EGTA in the pipette solution or 1 mM TEA in the external solution caused a reduction in the level of outward current evoked by metabolic inhibition. Single channels from on-cell patches revealed a substantial increase in activity of 135 pS (NP_o of 0.028 to 0.362 with cyanide) and 120 pS (NP_o of 0.13 to 1.61 with DNP) voltage-dependent channels under physiological recording conditions. These pharmacological and biophysical properties indicate that the dominant outward K^+ current affected by both pinacidil and metabolic inhibition using whole-cell and on-cell patch recording conditions in portal vein myocytes is produced by large conductance Ca^{2+}-activated K^+ channels.

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CONTENTS

LIST OF FIGURES AND ILLUSTRATIONS

PINACIDIL

AIM OF THE STUDY

In recent years there has been tremendous effort in the search for an answer to the question of how and what is the mechanism of action of potassium channel openers, the newest class of antihypertensive agents. These drugs cause hyperpolarization and cellular relaxation in smooth muscle. Hyperpolarization and blood vessel relaxation also result when smooth muscle tissue is metabolically depressed, such as occurs during ischemia, hypoxia or treatment with metabolic poisons. In cardiac muscle the action of potassium channel openers and metabolic inhibitors are known to occur through the same mechanism, and in smooth muscle it may be possible that the relaxation by these two processes are also mediated by a single mechanism. However, as of yet, no consensus has been reached regarding the exact type of channel(s) that is(are) affected by either K^+ channel openers or metabolic inhibition in smooth muscle. An exact understanding of the mechanism of relaxation of vascular smooth muscle may pave a road to new and perhaps more specific tools in the treatment of hypertension. It was the aim of this study to investigate the characteristics of the outward currents and single channels affected by both a potassium channel opener and metabolic inhibition. Particular attention was given to the responses of single portal vein myocytes to the potassium channel opener pinacidil.

INTRODUCTION

A high level of blood pressure, or hypertension, is known to correlate with increased risk of heart disease (Guyton 1991). The combined factors of fluid volume and blood vessel diameter contribute to the pressure load of the cardiovascular system, the latter determined by the level of sustained contraction in the smooth muscle tissue of the vessel wall. Relaxation of vascular tone, therefore, is one strategy employed to reduce the overall level of blood pressure in the body.

In recent years a new class of vascular dilating drugs (vasodilators) called potassium channel openers has been developed which cause relaxation of smooth muscle in ways different from the more traditional treatments such as calcium (Ca^{2+}) channel blockers. This review examines the published scientific literature concerning the cellular effects of these new vasodilators, and specifically the different mechanisms by which they are proposed to cause smooth muscle relaxation. In order to understand how these compounds effect cellular relaxation by opening K^+ channels, a brief summary of the Ca^{2+} handling mechanisms pertaining to cellular contraction in vascular smooth muscle is now discussed.

Ca^{2+} AND SMOOTH MUSCLE CONTRACTION

All muscle uses Ca^{2+} in the process of contraction, and in smooth muscle, Ca^{2+} forms a structural complex with an intracellular protein, calmodulin. This complex activates a kinase which in turn phosphorylates a protein of the myosin light chain, permitting crossbridge formation between myosin heads and actin filaments resulting

in cellular contraction (Hai and Murphy 1988, Rembold and Murphy 1988, Somlyo 1985). Tone or steady-state contractile response has been interpreted as an index of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), with the assumption that Ca^{2+} acts as a trigger for the contractile process (Kamm and Stull 1985, Rembold and Murphy 1988).

Normally $[Ca^{2+}]_i$ in the cytosol is extremely low, and as such a 10,000 fold Ca^{2+} gradient exists across the sarcolemma (SL) and the sarcoplasmic reticulum (SR) (van Breemen and Saida 1989). Several strategies prevent the cytosol from equilibrating with the extracellular milieu but at the same time allow limited Ca^{2+} entry for excitation-contraction coupling. Ca^{2+} enters the cell through membrane spanning voltage or receptor operated Ca^{2+}-selective channels (Bolton 1979), or by the reverse mode of the Na^+/Ca^{2+} anti-porter (Sheu and Blaustein 1992). It is extruded against the concentration gradient by an adenosine-5′-triphosphate (ATP) utilizing sarcolemmal Ca^{2+} pump (van Breemen and Saida 1989), and by the forward mode of the Na^+/Ca^{2+} antiporter. Of the internal Ca^{2+} storage sites the most important is the SR which sequesters Ca^{2+} from the cytosol by an ATP-dependent transport mechanism, and which releases it back during cell activation (van Breemen and Saida 1989). This release is believed to be triggered by internal second messengers, such as inositol 1,4,5-triphosphate (IP_3) (Berridge and Irvine 1984, Hashimoto et al. 1985) and a Ca^{2+}-induced Ca^{2+} release mechanism (Ilino 1989). Additional Ca^{2+} sequestration is also thought to carried out by mitochondria but this contribution, if any, to the mechanics of cell contraction is still unknown (Sheu and Blaustein 1992).

SOURCE OF Ca^{2+} FOR CONTRACTION

The overall concentration of internal Ca^{2+} is a product of the balance between influx and extrusion of Ca^{2+} from the cell (Ashida and Blaustein 1987). Maintenance of tonic contraction is principally produced by influx of Ca^{2+} through voltage-sensitive (potential operated) or ligand-activated (receptor operated) Ca^{2+} channels (Karaki and Weiss 1988). Although release of Ca^{2+} from internal stores can cause transient cellular contractions (Hashimoto et al. 1986, Somlyo 1985), and persistent activation of the phosphatidylinositol (PI) cycle by sustained agonist stimulation can contribute to tonic contraction (Challiss et al. 1992), internal Ca^{2+} release is not considered to be important in long term maintenance of contraction, because the removal of extracellular Ca^{2+} has been show to result in the abolition of myogenic tone in smooth muscle preparations (Harder et al. 1987).

STEADY-STATE Ca^{2+} CURRENT

A combination of two separate properties of L-type Ca^{2+} channels (Cohen and Lederer 1987, Langton et al. 1989) is thought to be important in the maintenance of resting tone of smooth muscle. In figure (i), based on data from Langton et al. (1989), two curves display the nature of activation and inactivation of the voltage-sensitive Ca^{2+} channels as they pertain to membrane potential. Ca^{2+} channels open when membrane potential is depolarized (becomes less negative) into the so-called activation range, allowing Ca^{2+} to flow across the membrane into the cell down its electrochemical gradient. The number of activated channels, which relates to the absolute amount of current, depends on the degree of depolarization, as depicted by the sigmoidal activation curve in figure (i). Soon after activation has occurred, the

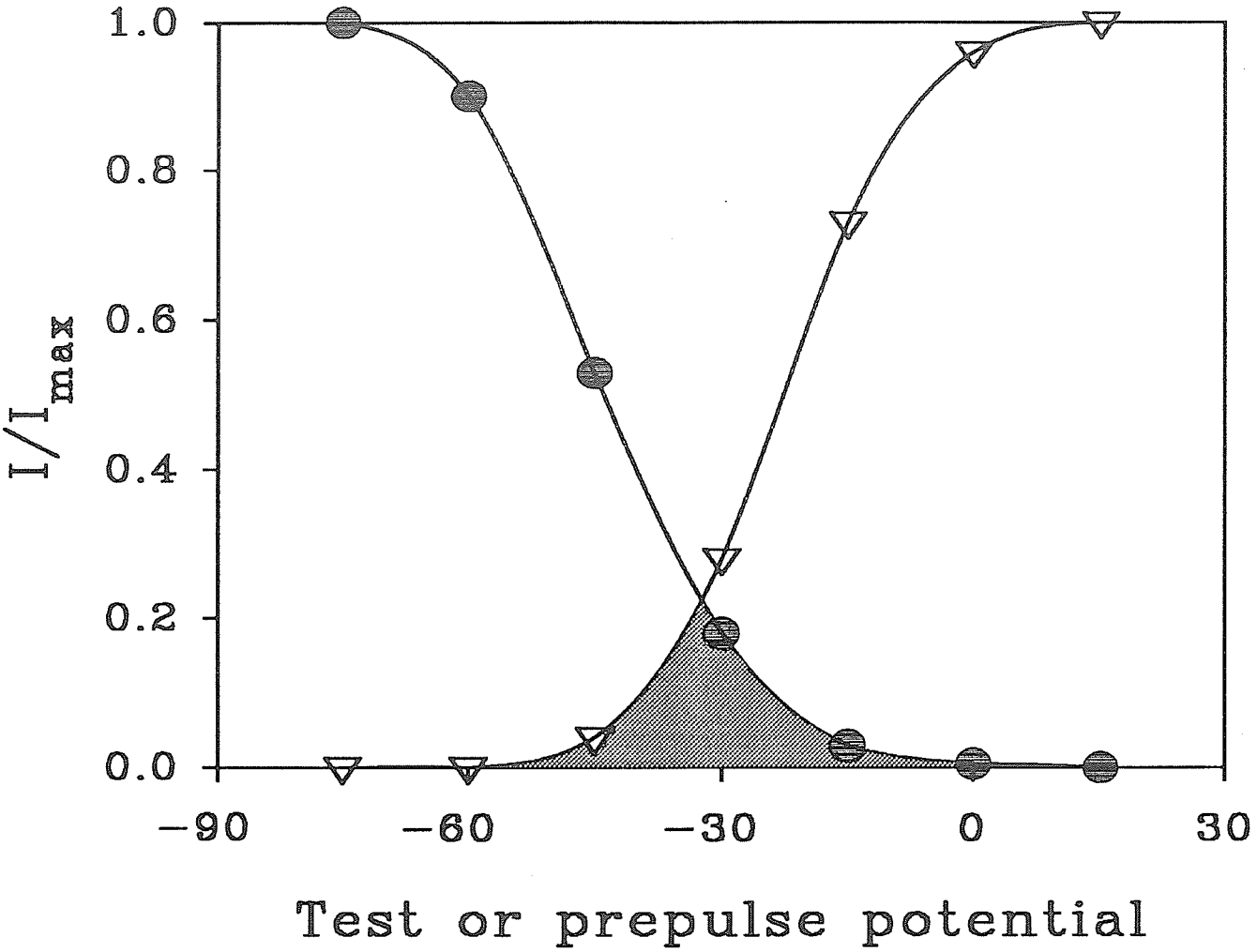

Figure (i): Activation and inactivation characteristics of Ca^{2+} current seen in vascular smooth muscle cells: concept of window current

Maximum level of current seen on activation (▽) of Ca^{2+} current after depolarizing to voltage indicated, and on inactivation (●) of Ca^{2+} current seen after holding at voltage indicated. Shaded area under two curves represents window current voltage range.

channels move into an inactivated and non-conducting state, particularly if membrane potential is held at the depolarized level for any length of time. The channels remain inactivated until the membrane is suitably repolarized (membrane potential returns to a more negative value). The percentage of channels that become inactivated depends on the degree of depolarization, as can be seen by the inactivation curve in figure (i). The concept of 'window' or steady-state Ca^{2+} current as described by Cohen and Lederer (1987) is easily understood when the two curves in figure (i) are examined. The area under the two curves represents the range of voltage where some, but not all channels may be activated, and where some but not all channels may be inactivated. Thus, within this range of potentials there is always a proportion of Ca^{2+} channels in the activated or open state, allowing a small but significant steady-state Ca^{2+} current to flow. In cardiac cells, window current is thought to play a major role in the maintenance of the action potential plateau (Lee et al. 1985). In smooth muscle the same properties of L-type Ca^{2+} channels have also been described (Langton et al. 1989) and are believed to contribute to the steady-state maintenance of elevated $[Ca^{2+}]_i$.

K^+ channels are a dominant conductance in smooth muscle, and play a major role in determining the value of resting membrane potential. K^+ channel openers (KCOs) have been shown to cause hyperpolarization of membrane potential in isolated smooth muscle myocytes (Leblanc et al. 1989, Noack et al. 1992) and intact tissue preparations (Hamilton and Weston 1989, Post et al. 1991, Weston and Edwards 1992). Relaxation of smooth muscle also occurs by metabolic poisoning (Mathew et al. 1991) or hypoxia (Moreland et al. 1991) which is also known to cause reductions in vascular resistance (Daut et al. 1990). Relaxation in these cases is considered to

occur through an increased level of K^+ channel activity (Silberberg and van Breemen 1992). The involvement of K^+ channels in these processes has led to the hypothesis that increased K^+ channel activity prevents Ca^{2+} influx by shifting membrane potential out of the range for voltage-dependent activation of L-type Ca^{2+} channels (Hamilton and Weston 1989, Weston and Edwards 1992), inhibiting the background steady-state window Ca^{2+} current and leading to a decrease in $[Ca^{2+}]_i$ and the abolition of myogenic tone (Nelson et al. 1990).

K^+ CHANNEL OPENERS

The mechanism whereby the vasodilating KCOs cause smooth muscle relaxation has been a subject of scientific interest for about the last 15 years. KCOs are a broad range of structurally different compounds that are grouped together by their ability to modulate the activity of sarcolemmal potassium selective channels in several tissue types (Cook et al. 1988). They include pinacidil (N-alkyl-N-cyano-pyridylguanidine), minoxidil-sulphate (2,4-diamino-6-piperidenyl-pyrimidine 3-oxide), nicorandil (2-nicotinamidoethyl nitrate), cromakalim or BRL 34915 ((±) 6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol), lemakalim or BRL 38227 (the negative enantiomer of cromakalim), aprikalim or RP 52891 (trans-(-)-N-methyl-2-(3-pyridyl)-2-tetrahydrothio-pyrancarbothiamide-1-oxide), celikalim or WAY 120,491, bimakalim or EMD 52692 (4-[1,2-dihydro-2-oxo-1-pyridyl]-2,2-dimethyl-2H-1-denzopyran-6-carbonitrite) and diazoxide. Many new versions of these drugs continue to be developed, such as Ro 31-6930 a variation of lemakalim which is up to 5 times more potent (Weston and Edwards 1992). The effects of cromakalim and its negative enantiomer lemakalim have been the main focus of the KCO research up until this

time.

Pinacidil, one of the first KCOs, was developed in Denmark in the early 1970s (Weston 1988), but it was not until the late 1970s that its vasodilating properties were appreciated (Petersen et al. 1978). Its effects are not centrally mediated and can still be observed in decerebrated cats (Petersen et al. 1978) or pithed rats (Olsen and Arrigoni-Martelli 1983, Richer et al. 1990). Pinacidil does not stimulate or inhibit α, ß, cholinergic or histaminergic receptors (Petersen et al. 1978), adenosine effects or prostaglandins (Olsen and Arrigoni-Martelli 1983, Kawashima and Liang 1985). Its actions are also endothelium independent and do not cause increases in intracellular cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) levels (Kauffman et al. 1986, Southerton et al. 1988, Gillespie and Sheng 1988).

A concurrent activation of guanylate cyclase with the vasoactive response of porcine and guinea-pig coronaries to nicorandil led to an early conclusion that the KCO mechanism was akin to nitrovasodilator drugs (Furukawa et al. 1981). However, there are differences between the actions of pinacidil and glyceryl trinitrate on mechanical and electrical activity (Bray et al. 1987). For example, relaxation due to nitrovasodilators is not associated with hyperpolarization unlike that of nicorandil (Taylor et al. 1988). For this reason KCOs have generally been considered to operate in a mode distinctly different from nitrovasodilators. Influx of $^{45}Ca^{2+}$ due to 128 mM K^+ depolarization was found to be significantly reduced by the dihydropyridine nifedipine but was unaffected by pinacidil (Mikkelsen and Pedersen 1982), evidence that pinacidil does not directly block voltage-sensitive Ca^{2+} channels. Although nicorandil can inhibit stimulated spike generation, spikes can be re-triggered at higher intensities of stimulation, demonstrating that Ca^{2+} channels can still be activated

(Sumimoto et al. 1987).

It was not until cromakalim was shown to increase K^+ conductance in papillary muscle (Cain and Metzler 1985) that K^+ was recognized to participate in the relaxation of smooth muscle by KCOs. Using the radio-isotope ^{86}Rb as a marker of K^+ efflux, cromakalim (Hamilton et al. 1986) and pinacidil (Southerton et al. 1988) were both found to enhance K^+ efflux, decrease membrane resistance, abolish spontaneous membrane activity and cause hyperpolarization of RMP close to E_K (calculated reversal potential for potassium) in rat portal vein. However these studies gave no indication of which K^+ channel(s) was(were) involved in the response to these drugs.

K^+ CHANNELS POSTULATED IN THE KCO RESPONSE

Much work has been produced since the discovery that K^+ efflux was a major participant in the smooth muscle relaxation process, particularly in the quest to reveal the identity of the channel responsible for the outward K^+ current. It has been firmly accepted that the action of KCOs in cardiac cells is through activation of adenosine-5'-triphosphate-sensitive K^+ channels (K_{ATP} channels). In smooth muscle, however, a consensus on the type of channel(s) activated has not yet been reached. The available data suggests the possible involvement of several types of K^+ channels in addition to K_{ATP}. The following section deals with the evidence surrounding the various ideas supporting the different kinds of channel thought to be modulated by KCOs to cause hyperpolarization in smooth muscle.

Adenosine-5'-triphosphate-sensitive K^+ channels: In single isolated cardiac myocytes, cromakalim or pinacidil in a temperature dependent manner (at 37 but much less at 22 °C) (Sanguinetti et al. 1988, Martin and Chinn 1990) cause

shortening of action potential duration similar to that seen in conditions of hypoxia or anoxia (Sanguinetti et al. 1988). These effects are reversed or inhibited by the anti-diabetic sulphonylurea, glibenclamide (also known as glyburide), a blocker of the glucose/ATP-sensitive K^+ channels in pancreatic ß-cells (Schmid-Antomarchi et al. 1987) and K_{ATP} channels in cardiac cells (Fosset et al. 1988). KCOs have been shown to abolish the negative slope of the inward rectifier K^+ current (Kakei et al. 1985, Iijima and Taira 1987, Osterrieder 1988) and also increase time- and voltage-independent steady-state outward K^+ current which is sensitive to internal concentrations of ATP (Escande et al. 1989, Arena and Kass 1989, Tseng and Hoffman 1990). The pinacidil-induced activation of K^+ channels in inside-out patches from cardiac myocytes may indicate a direct effect (Fan et al. 1990), although the temperature dependence of the effect suggests that an intermediate, perhaps enzymatic process, is still involved. These characteristics strongly indicate the involvement of K_{ATP} channels (Noma 1983) in the cardiac response to KCOs.

In contrast to cardiac myocytes, the type of K^+ channel(s) responsible for increased K^+ efflux and hyperpolarization in smooth muscle cells during exposure to KCOs is unclear. K_{ATP} channels have been observed in several other tissue types, including skeletal muscle (Spruce et al. 1985), pancreatic ß-cells (Cook and Hales 1984, Findlay et al. 1985) and, more recently an ATP-sensitive, voltage-independent K^+ channel that was blocked by glibenclamide and activated by cromakalim, was also observed in smooth muscle cells from the rabbit mesenteric artery (Standen et al. 1989). However the channels observed in this latter study appear to be distinctly different in several respects compared to K_{ATP} channels seen in other tissues. The conductance of the channel is larger (135 pS recorded with intracellular concentration

of 60 mM K^+, and extracellular concentration of 120 mM K^+), compared to those seen in cardiac and pancreatic tissue (between 50 and 80 pS in 140:140 K^+ and 20 pS using physiological concentrations), and although cromakalim has about a one hundred times higher potency than diazoxide as a vasodilator, at concentrations where it easily causes relaxation of smooth muscle, cromakalim is unable to evoke changes in blood glucose levels unlike diazoxide at that same dose (Quast and Cook 1989). These differences in specificity indicate a lack of a uniformity between K_{ATP} channels of pancreatic ß-cells and smooth muscle. Furthermore, galanin, a peptide which is known to activate K_{ATP} channels in pancreatic ß-cells, actually produces contraction of smooth muscle (Weston et al. 1990), completely the opposite that would be expected from the activation of K_{ATP} channels.

Aside from these discrepancies there is some evidence in support of the hypothesis that KCOs activate an ATP-sensitive K^+ channel in vascular smooth muscle. Lemakalim has been reported to enhance a Ca^{2+}-insensitive outward current in rabbit portal vein that was time- and voltage-insensitive, and inhibited by glibenclamide, but not affected by either 5 mM tetraethylammonium ion (TEA^+), or 4-aminopyridine (4-AP) (Russell et al. 1992). This suggests the involvement of an ATP-sensitive K^+ channel perhaps similar to that reported by Standen et al. (1989), and based on the lack of sensitivity to TEA^+ and 4-AP (see below) it was concluded that a Ca^{2+}-activated or delayed rectifier K^+ channel was not involved. Quast and Cook (1988) observed an increase in ^{86}Rb efflux by cromakalim which was sensitive to glibenclamide and in rabbit mesenteric arteries, an ATP-sensitive outward K^+ conductance, measured by ^{42}K efflux, was enhanced by cromakalim and inhibited by glibenclamide, which alone had no effect on the background ^{42}K efflux in control cells,

suggesting that glibenclamide was specifically blocking the cromakalim induced outward current (Post and Jones 1991). Membrane potential changes of isolated smooth muscle cells by KCOs or by alterations of $[ATP]_i$ (flash photolysis-induced release of pipette dialysed "caged" ATP) (Clapp and Gurney 1992) occurred with a concurrent increase in outward current which was glibenclamide-sensitive.

Glibenclamide has been shown to inhibit the cromakalim-induced vasodilation, and alone does not significantly affect resting tone of rabbit aorta (Quast and Cook 1989). Similar results have also been made in rabbit cerebral arteries (Brayden and Nelson 1992) suggesting that the channels influenced by KCOs are not normally involved in the maintenance of resting tone. The fact that the actions of KCOs can be blocked by glibenclamide, has spawned a widely accepted notion that K_{ATP} channels must be the most likely targets of these drugs in smooth muscle (Buckingham et al. 1989) and a similar philosophy has been used in the design of many intact-tissue or animal experiments to 'demonstrate' the presence of K_{ATP} channels in smooth muscle (Hood et al. 1991, Daut et al. 1990). However, the concept that glibenclamide is a specific blocker of K_{ATP} channels is becoming doubtful as this issue is further examined. For example, in skeletal muscle Quasthoff et al. (1990) described two separate K^+ channels (ATP-sensitive and ATP-insensitive) both of which were found to be inhibited by glibenclamide. Also, the response of KCO-relaxed smooth muscle to glibenclamide occurs at a concentration dissimilar to that used to induce blockade of K_{ATP} channels in pancreatic cells, which again suggests the possibility of a different type of channel (Buckingham et al. 1989, Weston et al. 1990). Furthermore, it has been shown that glibenclamide can inhibit large conductance Ca^{2+}-activated K^+ channels in rat portal vein (Hu et al. 1990) and affect SR Ca^{2+} handling in rabbit portal

vein (Xiong et al. 1991). With this evidence it must be concluded that there is a reasonable doubt to the claim that glibenclamide is a specific blocker of K_{ATP} channels in every tissue.

Large conductance Ca^{2+}-activated K^+ channels: Large conductance K^+ channels with open probabilities that are influenced by internal Ca^{2+} concentration have been found in a wide variety of smooth muscle types (Bolton et al. 1985, Inoue et al. 1985, Benham et al. 1986, McCann and Welsh 1986, Singer and Walsh 1987, Yamamoto et al. 1989). Conductance sizes range between 200 and 350 pS, measured in membrane patches using symmetrical K^+ gradients, or 100 to 150 pS in physiological K^+ gradients. They can be blocked by non-specific K^+ channel blockers such as Ba^{2+} (Bolton et al. 1985) or specifically by low concentrations (0.1 - 1 mM) of extracellularly applied TEA^+ (Inoue et al. 1985, McCann and Welsh 1986, Ohya et al. 1987) and (1 - 100 nM) charybdotoxin (CTX), a polypeptide component of venom from the scorpion *Leirius quinquestriatus hebraeus* (Miller et al. 1985). It has been reported that the activity of these channels is inhibited by chelation of internal Ca^{2+} by EGTA (Ohya et al. 1987).

The possibility that the Ca^{2+}-activated large conductance K^+ channel (K_{Ca}) could be modulated by KCOs in smooth muscle was raised after Kreye and Weston (1986) indicated that the relaxant effect of cromakalim was Ca^{2+} dependent, a quality also noted by Stockbridge et al. (1991). Cromakalim caused membrane hyperpolarization and increased the opening probability of a 200 pS K^+ channel (symmetrical conditions) which was also blocked by application of a crude scorpion toxin presumably containing CTX (Kusano et al. 1987). Cromakalim also caused a decrease in the time constant for the closed state of 337 pS Ca^{2+}-sensitive K^+ channels incorporated in

reconstituted membranes of aortic smooth muscle (Gelband et al. 1989). Recently Carl et al. (1992) conducted a study on the effects of cromakalim and lemakalim on single channels in colon smooth muscle cells. In both cell-attached and excised patches a reversible increase in the open probability of 265 pS channels (symmetrical conditions) was observed with the addition of KCO. Under conditions when the channels were maximally activated, KCOs failed to further increase the overall level of channel activity, suggesting that these drugs were enhancing only K_{Ca}. The increases in channel activity were antagonized by low (0.2 mM) TEA^+ and also glibenclamide. Some reports suggest that the modulation of K_{Ca} channels by glibenclamide can only occur after previous activation by KCOs (Hu et al. 1990, Langton et al. 1991).

Only a few studies have specifically looked at the whole cell currents affected by KCOs. One such study reported the activation of a non-inactivating voltage-dependent outward current in rat portal vein that was enhanced by cromakalim and P1060, an active analogue of pinacidil, and blocked by both CTX or glibenclamide (Hu et al. 1990). A similar current in rat portal vein was also reported to be increased by lemakalim, and blocked by glibenclamide (Noack et al. 1992). Although the whole cell data in these two studies appear very similar, there is no agreement on the conductance of the single channels responsible for these currents, (251 pS - Hu et al. and 17 pS - Noack et al.).

Several reports did not show support for the involvement of K_{Ca} in the KCO-response of smooth muscle. For example, cromakalim had no effect on large conductance K^+ channels in isolated myocytes of mesenteric arteries (Langton et al. 1991) and CTX could not inhibit the cromakalim-induced ^{86}Rb efflux in rabbit aorta

(Strong et al. 1989) or minoxidil-induced relaxation in rat portal vein, which was inhibited by glibenclamide (Winquist et al. 1989). Challenging this and using the same tissue, Lodge et al. (1991) described a sensitivity of the celikalim induced increase in ^{86}Rb efflux and tissue relaxation with low concentrations (I_c50 = 0.38 mM) of TEA^+, which is known to specifically inhibit K_{Ca} in other preparations (Inoue et al. 1985, Ohya et al. 1987, Langton et al. 1991), as well as with glibenclamide. In cerebral arteries, CTX and another scorpion toxin iberiotoxin (IBX), induced vasoconstriction pointing to the involvement of K_{Ca} channels in the maintenance of physiological resting tone (Brayden and Nelson 1992). However in this study, even in the presence of CTX or IBX, KCOs were still able to effect some vasodilation suggesting the involvement of channels or mechanisms other than the activation of K_{Ca}.

Small conductance Ca^{2+}-activated K^+ channels: A smaller conductance (10 - 15 pS) Ca^{2+}-sensitive K^+ channel is also present in smooth muscle (Inoue et al. 1985, Blatz and Magleby 1986, Isenberg and Klöckner 1986), and appears not to be involved in the cromakalim elicited hyperpolarization, as the specific blocker of this channel, apamin (Blatz and Magleby 1984) which is a component of bee venom (Habermann 1972), was ineffective in altering the cromakalim response (Weir and Weston 1986, Gillespie and Sheng 1988, Winquist et al. 1989, Okabe et al. 1990). However, Kajioka et al. (1990) demonstrated that a Ca^{2+}-dependent nicorandil-induced outward current in rat portal vein was not sensitive to 1 mM TEA^+ consistent with the involvement of a small conductance Ca^{2+}-sensitive K^+ channel. The voltage-dependent small conductance channel observed by Noack et al. (1992) may also fall into this category.

Delayed Rectifier K^+ current: Delayed rectifier K^+ current in smooth muscle is

a time- and voltage-dependent current which inactivates slowly over time (Beech and Bolton 1989a, Okabe et al. 1987), similar to that seen in other tissues (Hodgkin and Huxley 1952), and preferentially inhibited by 4-AP (Beech and Bolton 1989a). The main support for modulation of this current by KCOs comes from studies by Beech and Bolton, who observed differences in sensitivities of the inhibition by various K^+ channel blockers of cromakalim-enhanced current, compared to K_{Ca} current in these cells. The inhibition by 4-AP of only the former, led to the conclusion that the cromakalim-activated outward current was carried by the small conductance (4 - 7 pS) delayed rectifier channel (Beech and Bolton 1989b). However, that current was also inhibited by glibenclamide, a result the authors could not explain. Modulation of the delayed rectifier current has also been suggested by Nakao & Bolton (1991), who observed no effect of cromakalim or glibenclamide on a 55 pS CTX- and 5 mM TEA^+-sensitive K^+ channel, but did observe modulation of a 7.5 pS Ca^{2+}-sensitive and ATP-insensitive channel by the KCO. Furthermore, a cromakalim-induced current sensitive - to low concentrations of 4-AP and only to higher concentrations of TEA^+ (Wilson 1987), and a nicorandil-induced, Ca^{2+}-sensitive current also only inhibited by 10 mM TEA^+ or 4-AP (Kajioka et al. 1990), give further support to the concept that the delayed rectifier channel may be affected by KCOs.

ATP and/or Ca^{2+} Sensitive K^+ channel: Other channel types that do not completely fall into the above categories have been reported to be involved in the KCO effect. Kajioka et al. (1990) investigated the nicorandil induced Ca^{2+}-sensitive increase in outward current in rat portal vein cells and reported the presence of two conductances, a small 10 pS channel, blocked by 4-AP, but also inhibited by > 1 mM internal free ATP (but not by Mg-ATP as in the case of cardiac and pancreatic K_{ATP}

channels), and a larger 132 pS channel which was sensitive to low TEA^+. In addition, both channels proved sensitive to intracellular Ca^{2+} concentration, but only the activity of the small channel was modulated by nicorandil. The small conductance K^+ channel described here has some properties similar to those reported by Beech and Bolton (1989b), but also other additional properties not described by them. In rabbit portal vein a 150 pS channel was inhibited by < 1 mM TEA^+ or 50 nM CTX but was not affected by extremely high concentrations of pinacidil (500 μM), unlike a 15 pS channel which was activated by 100 μM pinacidil and inhibited by 100 μM glibenclamide (Kajioka et al. 1991). The activity of this small channel was also dependent on the presence of guanosine-5'-diphosphate (GDP) or guanosine-5'-triphosphate (GTP), as channels in excised patches showed no modulation by pinacidil unless GDP or GTP were present.

Multiple channel types affected: The idea that several channel types may be simultaneously modulated by KCOs has also been suggested. Due to differences in the concentrations of cromakalim needed to produce ^{42}K efflux and ^{86}Rb efflux in the same tissue it is argued that ^{86}Rb passes through a channel only activated by higher concentrations (>10 mM) of cromakalim, unlike ^{42}K (Bray and Quast 1991).

SECONDARY MECHANISMS OF VASODILATION INDUCED BY KCOs

There is increasing evidence that relaxation by KCOs may involve alternative intracellular mechanisms other than opening of K^+ channels. As described above, the earliest investigations into the action of nicorandil suggested a concurrent increase of guanylate cyclase activity (Furukawa et al. 1981) but the later comparison of pinacidil and glyceryl trinitrate which displayed differences in modes of action (Bray et al.

1987) may have diverted interest in other possible non-hyperpolarizing relaxation mechanisms. Previous reports indicated no change in either cAMP or cGMP concentrations by pinacidil or cromakalim (Kauffman et al. 1986, Southerton et al. 1988, Coldwell and Howlett 1987), however, suggestions that alternative mechanisms of relaxation do exist continue to emerge. For instance, although it is thought that nicorandil does not stimulate cGMP production to the same extent as the nitrovasodilators (Newgreen et al. 1988) the enhancement of cGMP by nicorandil is thought to be responsible for the drugs relaxation of tissue depolarized in high (80 mM) KCl (Sumimoto et al. 1987, Hamilton and Weston 1989). Increased intracellular cGMP is known to activate cGMP-dependent protein kinase which in turn activates Ca^{2+}-ATPase to reduce $[Ca^{2+}]_i$ (Vroxlis et al. 1988, Sumimoto et al. 1987). This may well contribute to a sizeable portion of nicorandil's action and it is possible that other KCOs may have a similar, although not so pronounced effect on intracellular cGMP levels. Indeed, pinacidil has been shown to relax 80 mM KCl depolarized smooth muscle tissue by lowering $[Ca^{2+}]_i$ (Nakajima et al. 1989) occurring without any alteration to membrane potential (Nakoa et al. 1988). Cromakalim can also relax low (20 mM) KCl depolarized muscle tissue (Newgreen et al. 1989), an effect antagonised by glibenclamide. Higher concentrations (100 μM) of cromakalim and pinacidil are required to relax contractions caused by 80 mM KCl and at these concentrations glibenclamide is unable to block their effect, suggesting the involvement of a separate (glibenclamide-insensitive) mechanism.

Several groups have reported that KCOs can induce alterations in the internal Ca^{2+} handling of the SR. In Ca^{2+}-free solutions cromakalim inhibited both the refilling and release of Ca^{2+} from the noradrenaline-sensitive intracellular stores in rabbit aorta

(Bray et al. 1991). In rabbit airway smooth muscle the presence of lemakalim decreased the rate of SR Ca^{2+} sequestration. This was demonstrated using ^{45}Ca as a marker of Ca^{2+} uptake, and was antagonized by glibenclamide (Chopra et al. 1992). At higher concentrations, glibenclamide itself appeared to reduce Ca^{2+} uptake and cause a slow release of Ca^{2+} from the SR (Chopra et al. 1992, Xiong 1991). The spontaneous release of Ca^{2+} from the SR (seen as spontaneous transient outward currents or S.T.O.C.s (Benham and Bolton 1986)) by pinacidil (Xiong et al. 1991) is perhaps evidence that pinacidil has two completely different effects within the cell, activation of membrane outward current, and inhibition of SR Ca^{2+} release. It has been hypothesized that SR membrane contains K^+ channels which function to balance the charge differences of extruding Ca^{2+} (Somlyo et al. 1981) and a reduction of K^+ conductance in the SR has been shown to increase the level of releasable Ca^{2+} from the SR (Fink and Stephenson 1987), and an increase in SR K^+ conductance may be expected to reduce Ca^{2+} release, as has been shown (Chopra et al. 1992, Xiong et al. 1991). Therefore it may be hypothesized that pinacidil can reduce internal Ca^{2+} release and uptake, through an increase in SR K^+ channel conductance, thereby decreasing the level of S.T.O.C. activity, but at the same time cause parallel increase in the K^+ conductance of the plasma membrane and hyperpolarization.

Turn over of sarcolemmal phosphatidylinositol (PI) by histamine or carbachol has also been demonstrated to be depressed by lemakalim, antagonized by glibenclamide, and not due to an increased accumulation of cyclic nucleotides (Challiss et al. 1992). The fact that the Ca^{2+} channel blocker, nitrendipine, can cause a similar inhibition of PI metabolism, and because cromakalim or pinacidil both failed to inhibit PI turnover in 65 or 128 mM KCl depolarized tissue (Itoh et al. 1992), leads to the

conclusion that prevention of Ca^{2+} entry by membrane hyperpolarization is the only factor interfering with membrane PI turnover.

Some KCOs have been demonstrated to interfere with the voltage-sensitive Ca^{2+}-channels in the membrane of smooth muscle cells. A decrease of Ca^{2+} current was reported for minoxidil-sulphate (Wilde and Hume 1987, Leblanc et al. 1989), cromakalim (Okabe et al. 1990, Post et al. 1991) and pinacidil (Nakashima et al. 1990). However, even at high concentrations these KCOs did not block Ca^{2+} current completely. Contrary to these observations, several groups have reported no antagonism of Ca^{2+} currents by KCOs (Mikkelsen & Pedersen 1982, Smirnov et al. 1992, Russell et al. 1992) and, when it occurs, the inhibition may be related to the type of KCO used since Post et al. (1991) saw no effect of lemakalim on the Ca^{2+} current unlike cromakalim. Modulation of other channel types by KCOs has been suggested, for example inhibition of Cl^- channels by pinacidil (Videbaek et al. 1990), but such reports are not prominent in the literature.

EFFECTS OF METABOLIC INHIBITION ON K^+ CONDUCTANCES

In cardiac tissue conditions such as hypoxia or anoxia, reduce the availability of O_2, cause a rapid reduction in developed tension (Allen and Orchard 1983) combined with a shortening of the action potential duration (Carmeliet 1978). Although ultimately the result of O_2 deprivation leads to a hypoxic contraction, the early contractile failure caused by hypoxia, cyanide or 2,4-dinitrophenol (DNP) (inhibition of only oxidative phosphorylation) appear to be as a result of a Ca^{2+}-independent uncoupling of the excitation-contraction mechanism. This is probably due to falling intracellular pH which occurs when there is a greater demand for glycolytic

ATP leading to enhanced lactate production. However when both oxidative phosphorylation and glycolysis are inhibited the early fall in developed tension appears to correlate with reduced internal Ca^{2+} concentration (Allen and Orchard 1984). Shortening action potential duration is also seen with complete metabolic inhibition and in both cases activation of ATP-sensitive K^+ channels has been shown to be primarily responsible for this (Noma 1983, Benndorf et al. 1991, Lederer et al. 1989). Although there has been some controversy over whether changes in intracellular metabolites (other than ATP) or inhibition of electrical excitability due to falling ATP levels (activation of K^+ channels) actually provides a greater influence on early contractile failure than the other (Koretsune and Marban 1990), it seems clear, at least in the case of complete metabolic inhibition in rat cardiac cells, that prevention of Ca^{2+} entry due to clamping of membrane potential near E_K after the activation of ATP-sensitive K^+ channels appears to be a dominant factor (Lederer et al. 1989).

In smooth muscle the actions of hypoxia or metabolic inhibition produce similar effects in developed tension or tone (Moreland et al. 1991). The involvement of K^+ in the relaxation response to hypoxia or MI is evident from studies where elevation of extracellular K^+ reversed the effect of MI on myogenic tone in rat portal vein (Sigurdsson and Grampp 1981) as well as the hyperpolarization seen after onset of hypoxia in cerebral arteries (Bonnet et al. 1991). As with the investigations of KCOs in smooth muscle, a response to glibenclamide has been used as evidence of the participation of the K_{ATP} channel in the changes induced by hypoxia (Daut et al. 1990) even although the nature of the currents or channels had not been explored.

As the actions of pinacidil, lemakalim or MI are all inhibited by glibenclamide it has been assumed that these effects are caused by modifications of the same channel

(Lydrup and Hellstrand 1991, Silberberg and van Breemen 1992). However in the heart complete block of the effects of high concentrations (300 μM) of cromakalim can be achieved only by 3 μM glibenclamide, while in cardiac cells treated with DNP it requires only 50 nM glibenclamide to partially block the effect (Fosset et al. 1988), suggesting that KCOs and MI do not cause the onset of outward current by identical means. Only one report to date states that hypoxia-mediated relaxation in smooth muscle is not inhibited by glibenclamide (Rodman et al. 1990).

More specific analysis of the nature of the MI-induced hyperpolarizing K^+ current in isolated myocytes of the rat mesenteric artery by Silberberg and van Breemen (1992) revealed a time- and voltage-insensitive current that was blocked by glibenclamide. However, the possible involvement of K_{Ca} channels in the effect of MI was minimized by the use of 5 mM TEA^+ and an absence of Ca^{2+} in the extracellular solution. Under these conditions, the action of lemakalim or MI on outward K^+ current were very similar.

In smooth muscle, a rise in $[Ca^{2+}]_i$ is known to occur as a consequence of MI (van Breemen et al. 1975), possibly due to the failure of ATP-dependent Ca^{2+} extrusion mechanisms. This rise in $[Ca^{2+}]_i$ may consequently trigger a massive increase in the open probability of K_{Ca} resulting in hyperpolarization and relaxation. Reductions in intracellular ATP are also hypothesized to directly affect K_{ATP} channels in smooth muscle. However Klöckner et al. (1992) investigated the effects of ATP on K_{Ca} channels in isolated patches and concluded that in the whole-cell situation, ATP could inhibit the activity of K_{Ca} channels due to the enhanced sequestration of $[Ca^{2+}]_i$ and therefore ATP directly or indirectly could cause alterations in activity of more that one type of single channel. It would therefore follow that a decrease in the

sequestration of $[Ca^{2+}]_i$ by a reduction in $[ATP]_i$ could enhance the activity of K_{Ca} channels.

Alternative mechanisms of muscle relaxation have also been hypothesized in the action of MI. Hypoxia may stimulate a $[Ca^{2+}]_i$ lowering mechanism without affecting membrane potential, as reductions in $[Ca^{2+}]_i$ during hypoxia were observed in smooth muscle preparations bathed in 122 mM K^+ solution (Pearce et al. 1992). As of yet, no studies have been published that implicate the involvement of any K^+ channel other than K_{ATP} in the relaxation action of MI.

RATIONALE FOR THE STUDY

The preceding review has attempted to touch upon the entire scope of KCO research published to date. It particularly focused on the hypothesized mechanism of action of the KCOs to indirectly interfere with voltage-sensitive Ca^{2+} channels, reducing $[Ca^{2+}]_i$ and causing vascular relaxation. Evidence in support of the various types of K^+ channel postulated to be responsible for mediating the hyperpolarization were presented along with data in support of other additional, non-hyperpolarizing relaxation mechanisms. As much as possible the relatively scant data concerning the effects of metabolic inhibition in smooth muscle were provided.

The reports surrounding the actions of KCOs presented here are highly conflicting and leave the critical reader dissatisfied. In fact, as of yet, no general conclusion has been reached and no evidence in favour of one, or other channel type has gained sufficient ground to push the consensus in any direction. Moreover certain aspects of the studies reviewed here leave further questions to be answered and doubts remain over some of the conclusions reached. For example, Beech and Bolton (1989b) use one piece of evidence, the fact that 4-AP appeared to inhibit the cromakalim induced current to a higher degree that the other K^+ channel blockers used, to conclude that delayed rectifier channels are activated by KCOs. They also used debatable evidence to rule out the participation of other types of channel, while reporting an inhibition of the KCO effect by glibenclamide, a blocker not known to influence the delayed rectifier channel. As of yet strong evidence to support their claim has not been forthcoming.

The keystone of the hypothesis that KCOs are modulating K_{ATP} channels in smooth muscle is the work by Standen et al. (1989). This study demonstrates the presence of ATP-sensitive K^+ channels activated by lowering of internal ATP concentrations or the application of cromakalim, and are antagonized by glibenclamide. In that study, similar to others supporting the involvement of K_{ATP} (Russell et al. 1992) the contribution of the large conductance Ca^{2+}-activated K^+ channel was minimized, by 5 mM EGTA before the application of the KCO, and therefore its contribution was not fully assessed. The K^+ concentrations used to record single channels were 60:120 mM, indicated a single channel conductance of about 135 pS, a size larger compared to K_{ATP} channels known to exist in other tissues. Moreover, even although their experiments were only conducted in excised patches they concluded that no other type of channel is modulated by cromakalim. Their conclusion may be criticized since modulation of channel activity by indirect mechanisms such as intracellular second messengers could not occur as they might under physiological conditions. The work of Gelband et al. (1989) suggested that reconstituted channels may be modulated by KCOs without the help of second messengers. However, as poor as the excised patch is as a model for the physiological situation, the reconstituted lipid membrane preparation must be regarded more critically, given the possible consequences of alterations in channel morphology, as well as the absence of intracellular co-factors on single channel activity. Other support for the K_{ATP} channel was presented by Clapp and Gurney (1992) who described a modulation of RMP by lemakalim or alterations in internal ATP concentration in smooth muscle myocytes, and using glibenclamide to inhibit these modulations, it was concluded that lemakalim was acting on a K_{ATP} channel, even though the specific

nature of the channels or currents activated by the KCO or ATP removal were not examined.

Regardless of the tissue in which it is employed, Standen et al. (1989) assumed glibenclamide to be a specific inhibitor of K_{ATP} channels. They used glibenclamide to support their hypothesis that not only were K_{ATP} channels present in smooth muscle, but that they were also responsible for the KCO relaxation seen in the intact tissue. The assumption that glibenclamide is a specific blocker of K_{ATP} channels has become very popular and has been used in many research studies as evidence supporting the conclusion that the vasodilator mechanism under study involved activation of K_{ATP} channels. Despite the widespread assumption of specificity for K_{ATP}, a comprehensive study of glibenclamide's effects on different membrane channels has not been preformed. However due to the many emerging reports which state that glibenclamide may have other effects not attributed to blockade of ATP-sensitive K^+ channels, any conclusions based only on the effects of glibenclamide must be questioned.

Few studies have systematically analyzed the whole-cell currents altered by KCOs. One such study by Hu et al. (1990) looked at the whole-cell currents affected by pinacidil and concluded that the enhanced current was passing through Ca^{2+}-activated K^+ channels. However the I-V relation described is positioned far to the right compared to the usual I-V characteristics seen for K_{Ca} channels (Inoue et al. 1985, Beech and Bolton 1989a) and K_{Ca} currents seen in other smooth muscle preparations (Cole and Sanders 1989). This was probably due to the fact that in these experiments 6 mM EGTA was included in the pipette solution. However this level of EGTA has already been reported to inhibit K_{Ca} at physiological membrane potentials (Ohya et al. 1987), so it is difficult to assess the type of current that they actually observed.

Investigations into the effects of metabolic inhibition in smooth muscle lack the scale of that done in cardiac tissue. As elsewhere, the techniques used to resolve the type of K^+ channel involved in MI vasodilation must be questioned. Daut et al. (1990) used glibenclamide to block the hypoxic or KCO relaxation of coronary artery and concluded the participation of K_{ATP} channels. However, aside from the previously mentioned doubts about the specificity of glibenclamide, there remains the question whether the fall in ATP levels seen by hypoxia in smooth muscle would be sufficient to activate K_{ATP} channels. Due to the similarities between KCOs and MI on cellular relaxation and increased K^+ efflux, an investigation into the nature of the channel(s) responsible for the MI hyperpolarization was warranted.

In the light of the many conflicting reports produced in these two areas, the effects of the vasodilator pinacidil or metabolic inhibitors were studied in the hope of clarifying questions raised by the earlier studies. Single cells were harvested from the rabbit portal vein due to the ease of preparation and previous use by other investigators. Particular attention was placed on the net whole-cell currents affected by pinacidil or metabolic inhibition, under conditions as much as possible resembling physiological conditions.

MATERIALS AND METHODS

SINGLE CELL ISOLATION

Smooth Muscle Cell Isolation: Smooth muscle cells were isolated from rabbit portal vein according to the method of Hume and Leblanc (1989). Rabbits of either sex were anaesthetized with CO_2 and sacrificed via cervical dislocation. The abdomen was opened, the hepatic portal vein removed and placed in Krebs solution (Krebs), containing (in mM) 120 NaCl, 25 $NaHCO_3$, 4.2 KCl, 0.6 KH_2PO_4, 1.2 $MgCl_2$, 1.8 $CaCl_2$, pH 7.4 (after bubbling with 95% O_2 : 5% CO_2). All solutions were made using pre-distilled water, deionized and filtered using a Barnstead Nanopure II filter system. The vein was cleaned of excess tissue and placed in a dissection-dish, filled with continuously bubbled Krebs. Adipose tissue and adventitia were dissected away under a binocular dissecting microscope (Wild M5APO) using fine scissors and forceps. The remaining tissue (predominantly a sheet of smooth muscle and endothelial cells) was cut into square pieces of approximately 2 mm and placed for 30 minutes into Ca^{2+}-free Krebs containing 0.1 mM ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA). The pieces were then placed in Krebs containing 10 μM Ca^{2+}, 184 units/ml collagenase (type 1A - Sigma Chemical Co., St Louis, MO., U.S.A.), and 0.1 mg/ml protease (type XXVII - Sigma) and gently bubbled at 31 - 33 °C for 20 minutes after which time they were repeatedly washed in Krebs containing 10 μM Ca^{2+}. Cells were encouraged to separate from the tissue pieces by gentle trituration. The liberated cells and tissue pieces were stored in 10 μM Ca^{2+} Krebs at 4 °C until use. The portal vein myocytes used in this study were found to possess similar

electrophysiological properties as previously described (Hume and Leblanc 1989).

Cardiac Myocyte Isolation: Guinea-pigs were anaesthetized with CO_2 and sacrificed by cervical dislocation. The heart was quickly removed and placed in well-aerated ice cold Krebs-Henseleit (K-H), containing (in mM) 120 NaCl, 25 $NaHCO_3$, 4.8 KCl, 1.2 Na_2HPO_4, 1.2 $MgSO_4$, 11 Glucose, 1.8 $CaCl_2$, pH 7.4. For a stabilization period of 10 - 15 minutes the heart was perfused retrogradely at a constant pressure with K-H at 37 °C by securing the cut end of the aorta to the outlet of a Langendorff apparatus after which time it was perfused with nominally Ca^{2+}-free K-H for 5 minutes before perfusion with Ca^{2+}-free K-H, containing 74.5 U/ml collagenase (Worthington Biochemical Corp., N.J., U.S.A.), for 45 min. The right ventricle was dissected free and placed in Ca^{2+}-free K-H, cut into square pieces of approximately 2 mm and further incubated in K-H containing 40.5 U/ml collagenase (Worthington) for 15 - 30 minutes at 37 °C. The tissue pieces were then washed and stored in 1.8 mM Ca^{2+} containing Krebs at room temperature and single cells were obtained when needed by gentle trituration.

EXPERIMENTAL PROCEDURE

Patch-Clamp Technique: The patch-clamp technique as described by Hamill et al. (1981) was the method used to record at ionic currents in the single isolated myocytes.

Described briefly, the patch-clamp technique, which is used to record both whole-cell current and voltage measurements as well as single channel currents, requires that a very fine tipped glass micro-electrode, connected to an electronic amplifier, is positioned on the membrane of an isolated living cell. Both whole-cell net

currents or single channel currents were measured in the following way;

Cells dispersed in solution, were placed in a chamber with a glass base (volume of 0.25 ml) set on the stage of a Nikon inverted TMS microscope (MFA 10300; Narishige Scientific Instrument Laboratory, Tokyo, Japan). After settling onto the glass cells were bathed with either a HEPES buffered Krebs containing (in mM) 120 NaCl, 3 $NaHCO_3$, 4.2 KCl, 1.2 KH_2PO_4, 0.5 $MgCl_2$, 11 Glucose, 1.8 $CaCl_2$, 10 HEPES (pH 7.4 corrected with NaOH) (for whole-cell or physiological gradient single channel) or a high K^+, nominally Ca^{2+} free solution containing (in mM) 140 KCl, 0.5 KH_2PO_4, 1 $MgCl_2$, 0.5 EGTA, 10 glucose, 5 HEPES (for symmetrical gradient single channel) at room temperature (20 - 22 °C) . Only cells of a spindle-shaped, optically-refractive and relaxed nature were chosen for experimentation.

Electrodes were pulled from capillary glass (Corning capillary tubing # 7052, inside diameter 1.2 mm, outside diameter 1.67 mm) on a Glass Microelectrode Puller PP-83 (Narishige) and the tips were heat polished using a Microforge MF 83 (Narishige) to an electrical resistance of between 2 - 5 MΩ. Electrodes were filled with intracellular pipette solution containing (in mM) 110 K-gluconate, 30 KCl, 0.5 $MgCl_2$, 5 HEPES, 0.1 EGTA, 5 Na_2ATP (whole-cell) or 140 KCl, 0.5 KH_2PO_4, 1 $MgCl_2$, 0.5 EGTA, 10 glucose (10 mM 2-deoxy-D-glucose was used instead of glucose in the metabolic inhibition experiments), 5 HEPES (single channel). Both solutions had a pH of 7.2 corrected by KOH. For single channel recording, electrodes were further coated with Sylgard silicon gel (Dow Corning, Midland, MI., U.S.A.) to reduce interference from external sources of electrical noise. Electrodes were placed in a holder connected to a patch-clamp amplifier (Axopatch 1-D; Axon Instruments Inc, Foster City, CA., U.S.A.) headstage (CV-4 1/100) and a chlorided-silver wire ran from the headstage

passing through the holder into the intracellular pipette solution. A second chlorided silver wire was placed in the bath and also connected to the headstage. The electrode, holder and headstage were suspended from a manual oil-driven micro-manipulator (MO-103N for fine and MN-1 for course manipulation; Narishige).

Using the microscope and manipulator, the pipette tip was manoeuvred onto the cell membrane (see plate on page 32 - demonstrating the image seen through the eye piece of the inverted microscope of a living portal vein myocyte before or during a patch, voltage or current clamp experiment) and negative pressure was applied inside the pipette by a syringe connected by plastic tubing to the electrode holder. A tight seal, having an electrical resistance of 1 - 10 GΩ, was formed between the glass and the cell membrane. A small depolarizing test pulse of 20 mV was applied to the pipette and the formation of a high resistance seal was monitored using an oscilloscope (Hitachi V-6025).

After seal formation, electrode capacitance was manually compensated by adjustment of the charging rate (fast and slow capacitance compensation). At this stage, single channel recording was performed when the membrane was still intact. The electrode was either left attached to the cell (on-cell patch) or was withdrawn from the cell, pulling off the electrode-attached patch and exposing the intracellular side for experimental manipulation (excised or inside-out patch).

For whole-cell recording, the membrane situated within the mouth of the pipette was ruptured either by applying further negative pressure inside the electrode, or by the use of the 'zap' feature of the amplifier which transmits a high frequency oscillating pulse through the electrode wire, resulting in membrane rupture. The amplitude of the transient capacitance evoked by the test pulse discloses the level of electrical access that the amplifier has to the cell's interior and this was closely

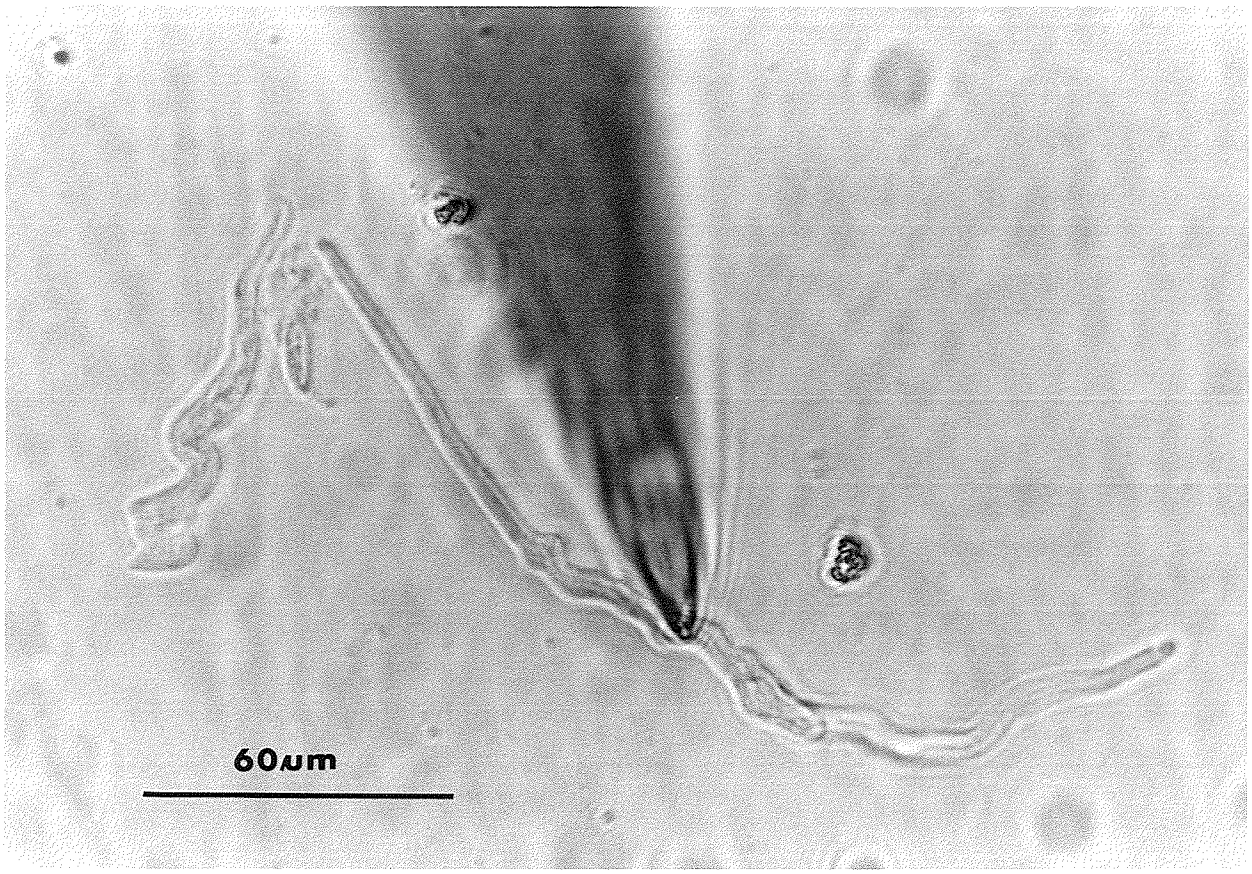

Plate: Smooth muscle myocyte under experimentation

This image depicts a cell which is being voltage clamped. The triangular shadow at the top of the plate is the out-of-focus tip of the electrode pipette and the pointed end is the open mouth of the pipette which, after the application of suction forms a strong, electrically resistant seal with the cell membrane. To the left of the cell lies the remnants of another cell which has contracted and is presumably dead. Cells that look like this are generally not suitable for experimentation.

monitored during the experiment. Deteriorating access was improved by further application of negative pressure within the electrode. Because charges applied to alter membrane potential in whole-cell mode must pass through the small pipette tip, significant voltage errors can occur as the magnitude of this series electrical resistance approaches that of the cell. To overcome this, the manually adjusted series-resistance circuitry of the amplifier can partially compensate for high pipette resistance or poor electrical access. In the whole-cell condition a certain amount of dialysis between the pipette and the cell makes it possible to manipulate the ionic contents of the cytosol or introduce drugs for experimental purposes.

Using pCLAMP or Axotape software (Axon Instruments Inc.) on an IBM compatible computer, voltage and current clamp protocols were delivered to the amplifier through an analogue to digital convertor, (TL-1-125 LabMaster Board, Axon Instruments Inc.). All measurements made by the amplifier were passed through an on-board 8-pole bessel filter at 1 KHz and stored on magnetic disks by the computer. Long duration experiments which create large amounts of data, especially single channel studies, were stored directly onto video tape through a PCM video adaptor (A.R. Vetter Co., Rebersburg, PA., U.S.A.) and selected portions of data were later digitized and analyzed by computer. Analysis of both whole-cell and single channel data was facilitated by the pCLAMP and Axotape software. Sources of background electrical interference (60 Hz) were attenuated by extensive earthing of all components, surrounding the microscope with a grounded faraday cage and using a DC source to power the microscope light. Access to the set up was achieved by a movable panel on the front of the cage. To maintain high resistance seals the microscope was mounted on a nitrogen-charged Micro-g floatation table (Technical Manufacturing Corp. MA., U.S.A.) to guard against background vibration.

Drugs and Compounds Used: The following drugs were used in this study. Stock solutions of pinacidil (a gift from Eli Lilly Co., Indianapolis, IN., U.S.A.) were made in polyethylene glycol (PEG); glibenclamide (Sigma Chemicals, St. Louis, MI., U.S.A.) in a 1:10 mixture of dimethyl sulphoxide (DMSO) and PEG; charybdotoxin (Research Biochemicals Inc., Natick, MA., U.S.A.) in 9% saline solution. All were diluted to appropriate concentrations in HEPES buffered Krebs bathing solution. 50 μM pinacidil was used unless otherwise indicated. Solvents did not alter electrical behaviour of the portal vein and cardiac myocytes at concentrations used. Tetraethylammonium chloride (TEA^+) 0.5 or 1.0 mM; 4-aminopyridine (4-AP) 10 mM; sodium cyanide (NaCN) 2 mM; 2,4-dinitrophenol (DNP) 50 μM (all from Sigma) were dissolved directly into the HEPES buffered Krebs. The different bathing solutions were stored in containers (50 ml syringe casings without plungers) positioned above the level of the chamber within the faraday cage, and conducted to the bath through lengths of plastic tubing which ran into a common tube before entering the bath. The gravity fed flow rate of 0.18 ml/min was controlled by a screw valve placed on the last section of tubing. In order to ensure fast change over between different solutions, all tubes were primed to a point just before their junction with the common tube. Turn over of solutions was facilitated by removing solution from the rear of the bath via a vacuum line attached to a trap-flask and vacuum pump.

Analysis of Single Channel Data: The changes to the activity levels of single channels recorded could not be assessed by comparison of the open probability values before and after exposure to the drugs, because in all cases more than one channel was present in the patch and multiple levels of activity were observed. Therefore the total amount of channel activity was used as an index of changes in open probability. The calculation of the product of the numbers of channels and open probability (NP_o)

has been described by Kajioka et al. (1991);

$$NP_o = (a_1 + 2a_2 + 3a_3 + ... + na_n)/(a_0 + a_1 + a_2 + ... + a_n)$$

where a_0, a_1, a_2, a_3, and a_n are the areas under each peak of the amplitude histogram with all channels closed, one open, and simultaneous openings of 2 to n channels, respectively.

RESULTS

PINACIDIL-INDUCED ALTERATIONS IN OUTWARD CONDUCTANCE

OBSERVATIONS IN SMOOTH MUSCLE

Pinacidil enhances a time-dependent K^+ current: Figure 1 illustrates the ability of pinacidil to reversibly hyperpolarize membrane potential in rabbit portal vein myocytes. Membrane potential changed by -18.5 ± 4.7 mV in 4 myocytes and the response occurred within 3 - 5 minutes of initial exposure. The influence of pinacidil (50 -100 μM) on macroscopic outward currents elicited by brief duration voltage clamp steps was initially studied. Figure 2 shows representative data in which 100 μM pinacidil increased outward whole-cell currents evoked by step voltage changes of 250 ms duration to a range of potentials from -80 to 0 mV from a holding potential of -60 mV. The increase in current occurred within 3 - 6 minutes exposure to the drug. Similar observations were made in 3 other myocytes and with 50 μM pinacidil in 4 myocytes.

By examination of the current traces in figure 2 it is evident that there may have been a simultaneous effect on two separate components of outward current; a time-dependent component, defined as the current which takes time to develop to its maximum level, and quasi-instantaneous component seen as the current occurring immediately after the capacitative transients had settled (arrows in Fig. 2). In these experiments it is not possible to confidently assign the term time-independent to the quasi-instantaneous current, as whether the current is passing through channels already in the open state, or through channels which rapidly activate due to the action of the drug, can not be ascertained. The increase in time-dependent outward current

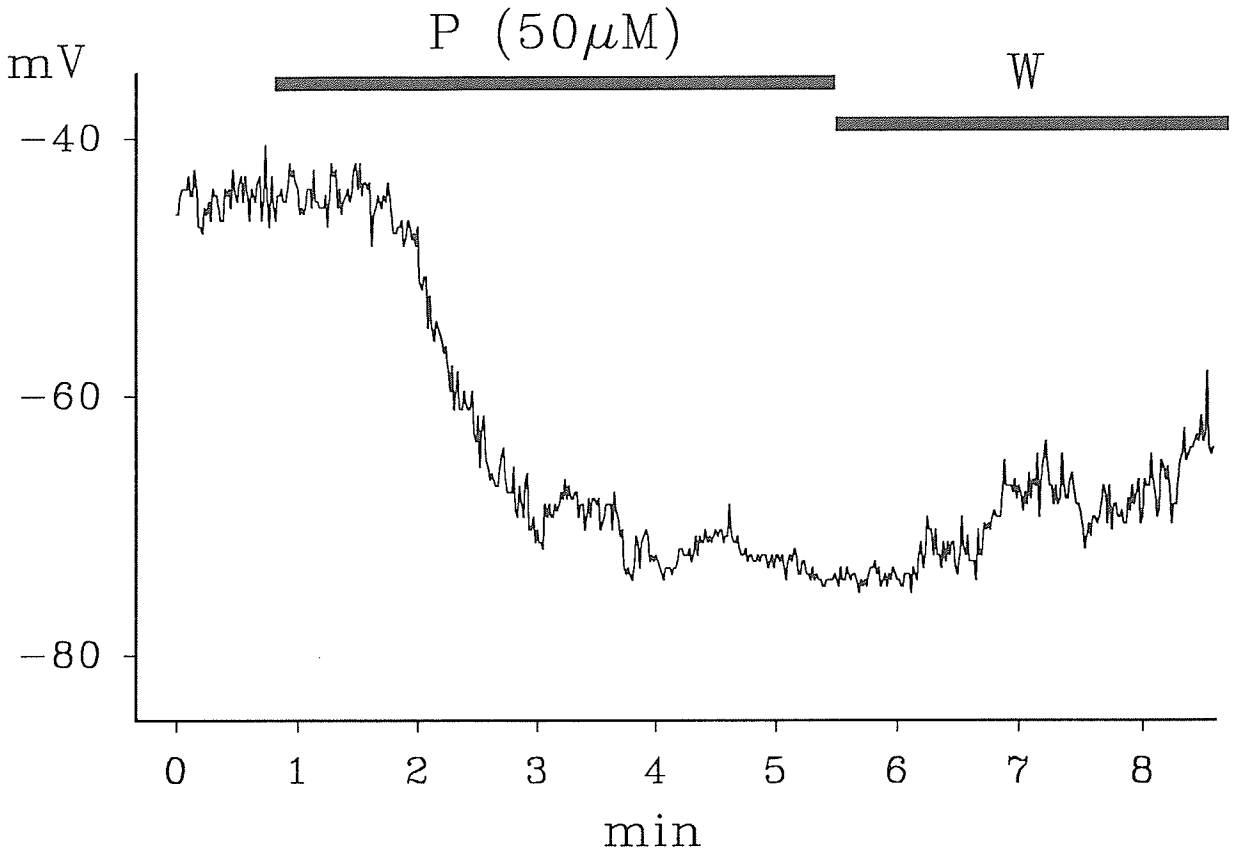

Figure 1: Effect of pinacidil on membrane potential in a portal vein myocyte.

Continuous membrane potential recording from a single portal vein myocyte during exposure to (P) and washout of (W) 50 μM pinacidil by whole-cell current clamp mode of patch-clamp (applied current = 0 pA).

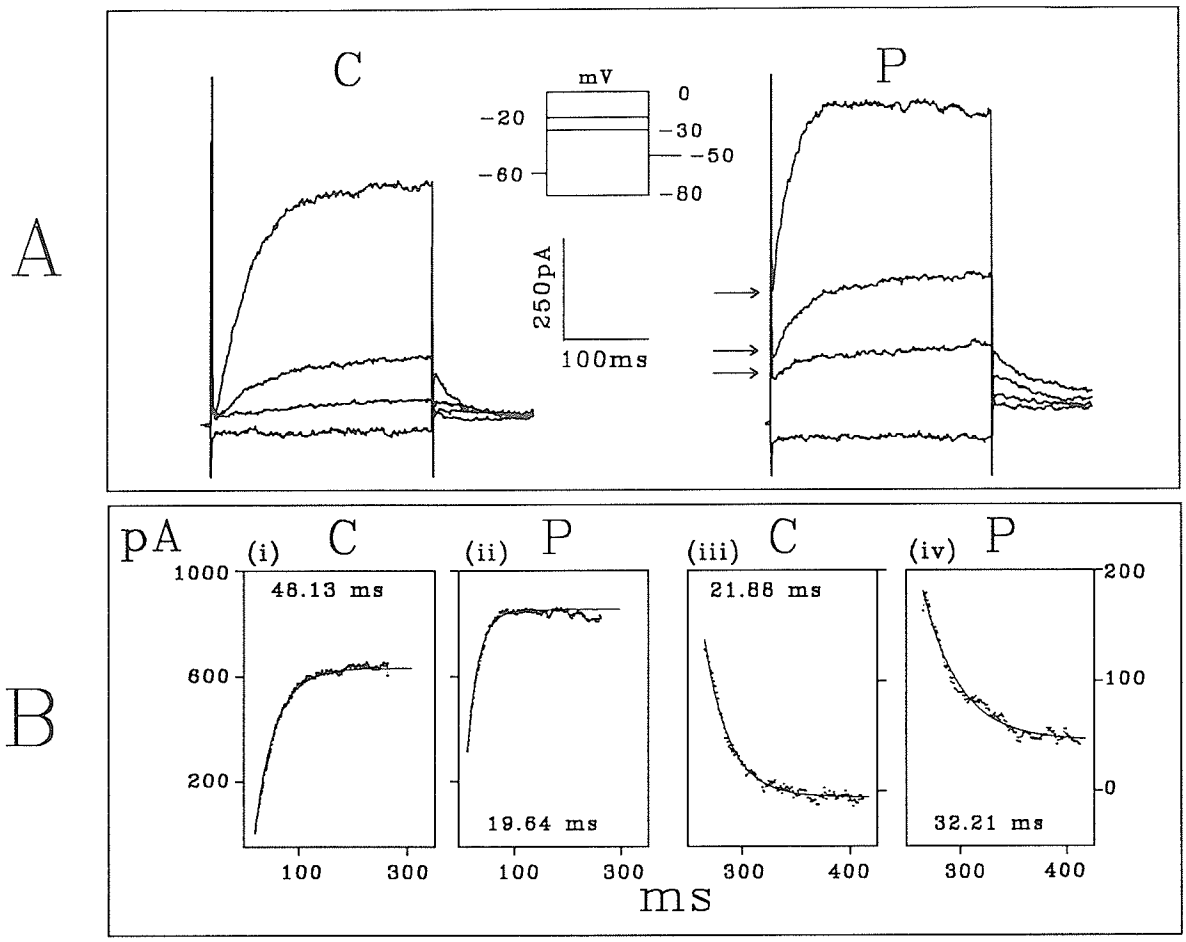

Figure 2: Effect of pinacidil on whole-cell current in a portal vein myocyte.

A: Families of membrane currents evoked under control conditions (C) and after 7 minutes exposure to 100 μM pinacidil (P) by application of voltage clamp protocol indicated. Arrows indicate magnitude of time-independent component at end of initial capacity transient. ***B:*** Fits of membrane current activation at 0 mV ((i) and (ii)) and tail current deactivation at -50 mV after steps to +20 mV ((iii) and (iv)). Solid lines are best fits to the data assuming the single time constant (τ) indicated in each panel.

was apparent both during the command pulse (note especially the current produced at the -20 mV step), and in the tail currents which occurred upon stepping back to -50 mV (Fig. 2). The activation and deactivation kinetics for outward current were both affected by pinacidil. Activation and deactivation time constants decreased and increased from 48 to 20 ms and 22 to 32 ms, respectively. Dissection of the various current components from the experiment shown in figure 2 are displayed in figure 3A. Pinacidil caused; a) a negative shift in the reversal potential closer to the calculated value for the equilibrium potential for K^+ (E_K; -89 mV in this study) and 2) an increase in outward current at all voltages between E_K and 0 mV, but did not alter the absolute outward current positive to 0 mV. This suggests that pinacidil shifted the voltage sensitivity of the current but not the maximum value. The negative shift in reversal potential and the negative value of the intersection point for the I-V relations obtained ± pinacidil (~ -80 mV) are consistent with an effect on a K^+ current. That pinacidil caused a negative shift in the voltage-dependence of activation of the time-dependent component is evident in figure 3B. Normalized peak tail currents (Fig. 3D) measured at -50 mV after steps to the range of potentials between -90 and +50 mV (V_t) were fit with a Boltzmann equation:

$$I = [1 + \exp((V_{1/2} - V_t)/k)]^{-1}$$

where $V_{1/2}$ and k are the voltage for half-maximal activation of current and the slope constant (k = 15), respectively. Pinacidil caused a shift of -10 mV in $V_{1/2}$ without a change in slope factor indicating a negative shift in the voltage-dependence of the K^+ current, without any major alteration in the conductance of the channels responsible for this current.

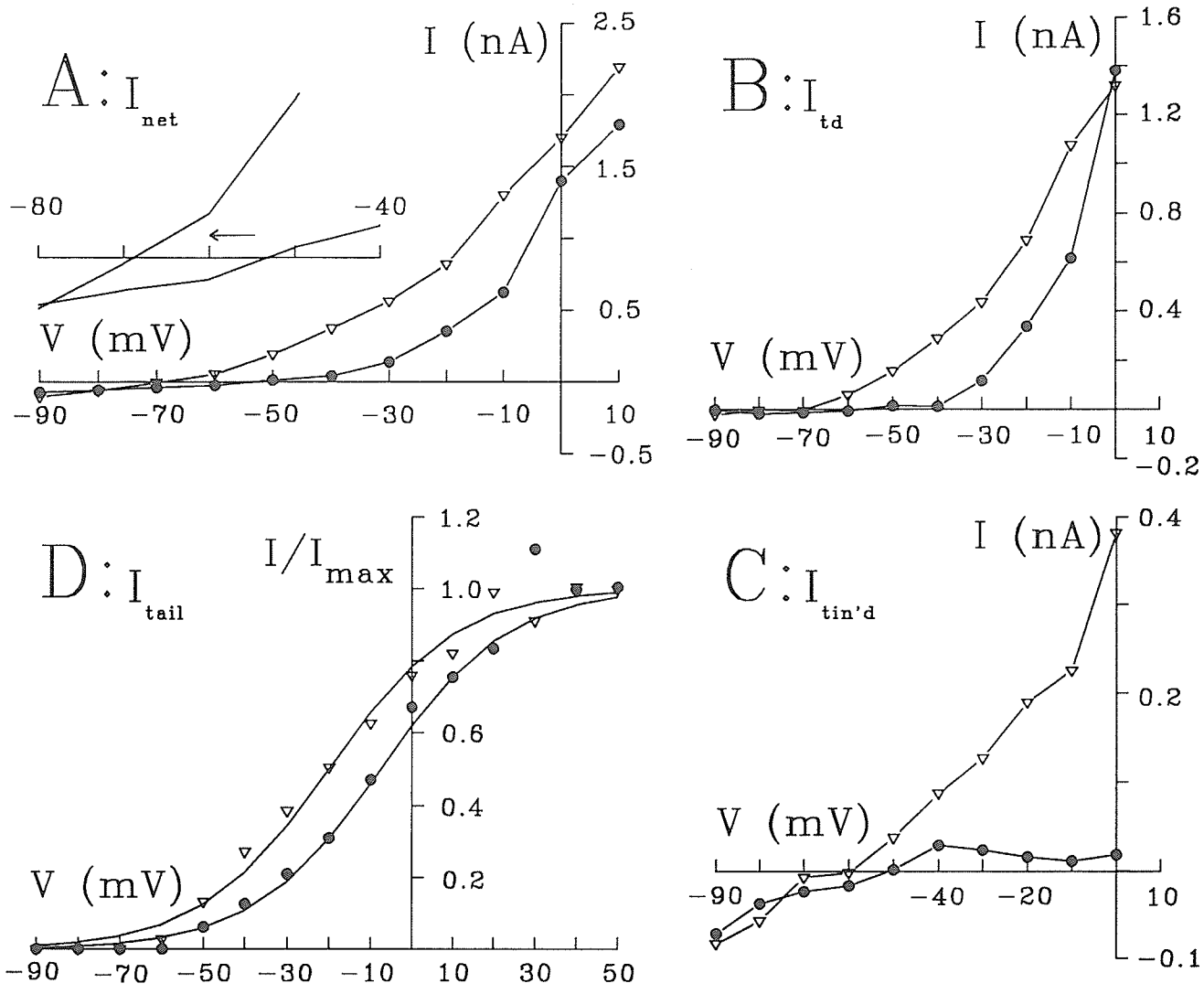

Figure 3: Effect of pinacidil on current-voltage relation for time-dependent and -independent components of whole-cell current.

A: I-V relation for peak whole-cell current (I_{net}) in absence (●) and presence (▽) of 100 μM pinacidil for myocyte in figure 2. Peak current measured at 250 ms during command pulse. Note the shift in reversal potential caused by pinacidil shown in inset. ***B:*** I-V relation for peak time-dependent component (I_{td}) in absence (●) and presence (▽) of pinacidil. Peak time-dependent current determined was at 250 ms during command pulse (i.e. peak net current minus time-independent component). ***C:*** I-V relation for time-independent component ($I_{tin'd}$) in absence (●) and presence (▽) of pinacidil.

continued.....

Figure 3: continued.....

Time-independent current amplitude was measured at end of capacity transient. ***D:*** Normalized tail current amplitude (I_{tail}) recorded at -50 mV versus voltage of command pulse in absence (●) and presence (▽) of pinacidil. The data points were fit with a Boltzmann relation (see text for additional details). Note the 10 mV negative shift in the activation curve without a coinciding change in slope factor after the addition of pinacidil.

Pinacidil enhances a quasi steady-state K^+ current: The I-V relation of the pinacidil-enhanced steady-state outward current, as is seen in figure 2 (arrows) is displayed in figure 3C. The increase in this component during step command pulses may indicate that pinacidil affected resting or background conductance(s) in portal vein myocytes. This possibility was explored through the use of a ramp protocol in which membrane potential was slowly varied between -95 and +10 mV over 8 seconds (13.125 mV/s) from a holding potential of -75 mV (Fig. 4A). The slow change in membrane potential permits sufficient time for time-dependent changes in membrane currents to occur and therefore the measured currents approximate those expected at each voltage under steady-state conditions. Pinacidil caused; a) an outward shift in holding current, b) an increase in quasi steady-state outward current, and c) a negative shift of the steady-state current in reversal potential towards E_K. Similar effects on steady-state current were observed within 3-6 minutes in 27 out of 32 myocytes exposed to pinacidil. Digital subtraction of control trace from that obtained in the presence of pinacidil (i.e. the pinacidil-sensitive component) reveal an outwardly rectifying difference current which reversed at -88 mV or E_K. The pinacidil-sensitive (difference) current demonstrated considerable "noise" at positive potentials for individual myocytes, suggesting pinacidil may have enhanced a large conductance channel. When the difference currents from *22 individual experiments were averaged, a non-linear I-V relation for the quasi steady-state current induced by pinacidil was evident (Fig. 4B). The non-linear nature of the difference current indicates pinacidil enhanced a voltage-dependent steady-state conductance.

[* 5 out of the 27 cells that displayed an unequivocal response to pinacidil could not be included in the mean difference current as they were originally recorded using ramps with a different rate of voltage change]

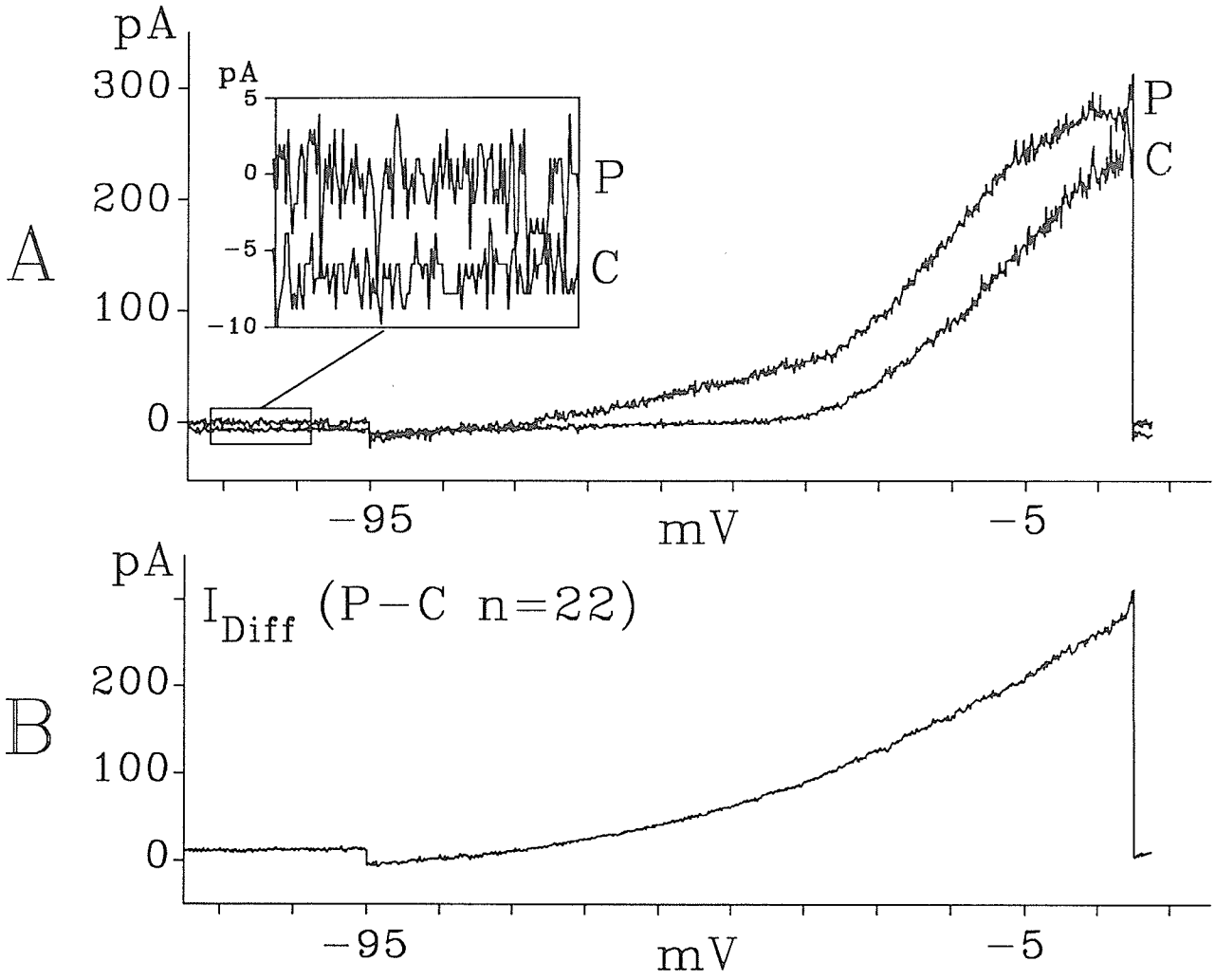

Figure 4: Effect of pinacidil on quasi steady-state whole-cell current.

A: Quasi steady-state membrane current evoked by an 8 second ramp of voltage between -95 and +10 mV from a holding potential of -75 mV in absence (C) and presence (P) of pinacidil. Note outward increase in holding current in the presence of pinacidil (inset) (traces obtained at 4 min drug exposure).

B: Average difference current from 22 myocytes obtained by digital subtraction of control traces from those recorded with pinacidil.

Characterization of K^+ current(s) enhanced by pinacidil: When Ca^{2+}-activated current components were inhibited by elevating EGTA in the pipette solution to chelate intracellular Ca^{2+}, no effect of pinacidil on outward current was observed. Figure 5 illustrates the absence of an increase in time-dependent current evoked during step command protocols, or steady-state outward current evoked by ramp protocols during exposure to pinacidil for times indicated in a myocyte dialysed with pipette solution containing 5 mM EGTA. A small decline in outward current was seen after extended periods of exposure to pinacidil and no change in holding current was evident. Similar results were obtained in three myocytes. The decrease in the inward component after the addition of pinacidil seen in figure 5A is most likely not attributed to the presence of pinacidil, as in almost all cases, when whole-cell access is achieved some decline or rundown of the Ca^{2+} current occurs over time.

Based on the sensitivity to intracellular Ca^{2+} chelation, pinacidil was also tested in the presence of external, a) TEA^+ at a low concentration (0.5 mM), and b) CTX (20 nM). Both of these manipulations may be expected to alter Ca^{2+}-activated K^+ current (K_{Ca}). TEA^+ experiments were conducted in three ways. a) whole-cell access was achieved before myocytes were exposed to TEA^+ (0.5 mM) and then to TEA^+ and pinacidil. b) myocytes were pre-exposed to TEA^+ (0.5 mM) for 10 - 20 minutes before whole-cell access was obtained and steady-state currents were then compared to those in the presence of both TEA^+ and pinacidil. c) Myocytes were briefly exposed to pinacidil followed by washout prior to brief TEA^+ (0.5 mM) treatment and a second exposure to pinacidil. Figure 6 demonstrates how pinacidil (50 μM) failed to significantly alter step or ramp-evoked outward current after prolonged pretreatment with 0.5 mM TEA^+. Similar effects were observed in 5 other cells. In one experiment, washout of TEA^+ provided evidence in the form of S.T.O.C.s (Benham and Bolton

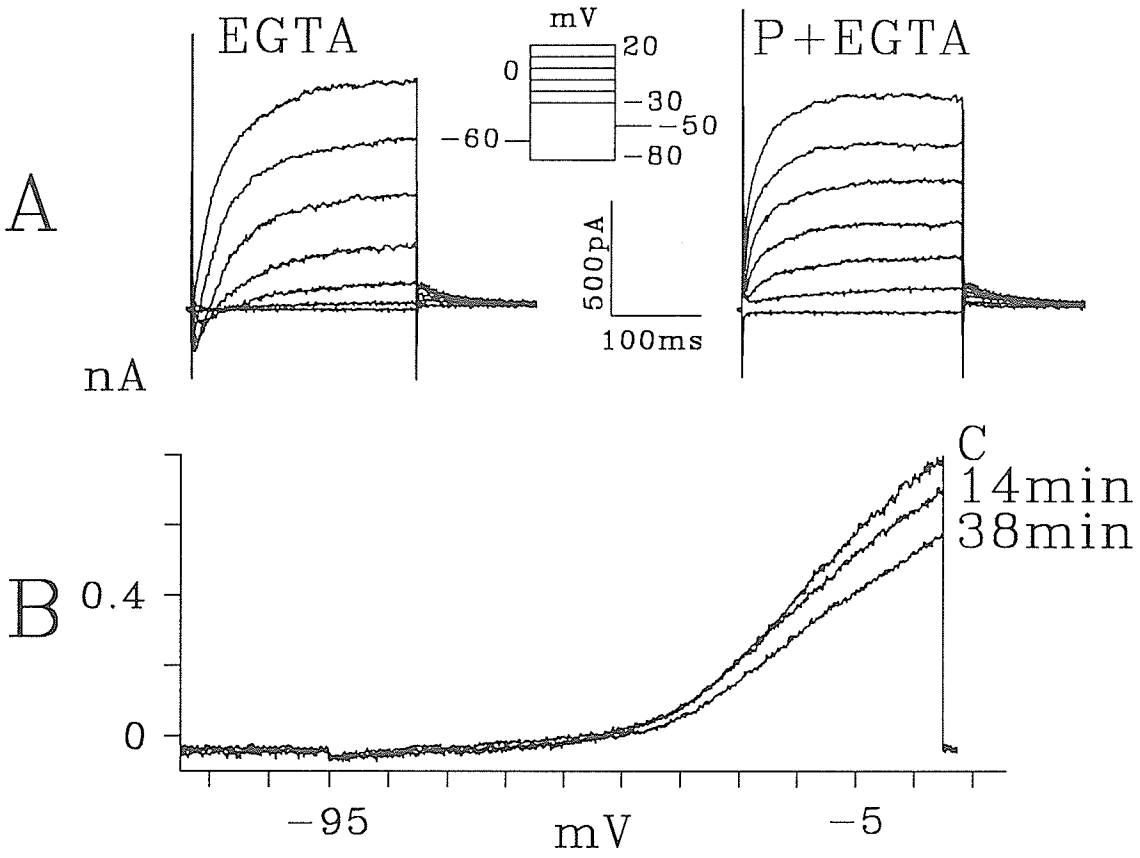

Figure 5: Effect of pinacidil on whole-cell current in a myocyte dialysed with 5 mM EGTA pipette solution.

A: Families of membrane currents recorded in the continued presence of 5 mM EGTA in the intracellular pipette solution, before (EGTA) and after (P+EGTA) the addition of 50 μM pinacidil, evoked by application of voltage clamp protocol indicated. ***B:*** Quasi steady-state current evoked by ramp protocol (see legend Fig. 4) in absence (C) and at 14 and 38 minute exposure to pinacidil (14min, 38min).

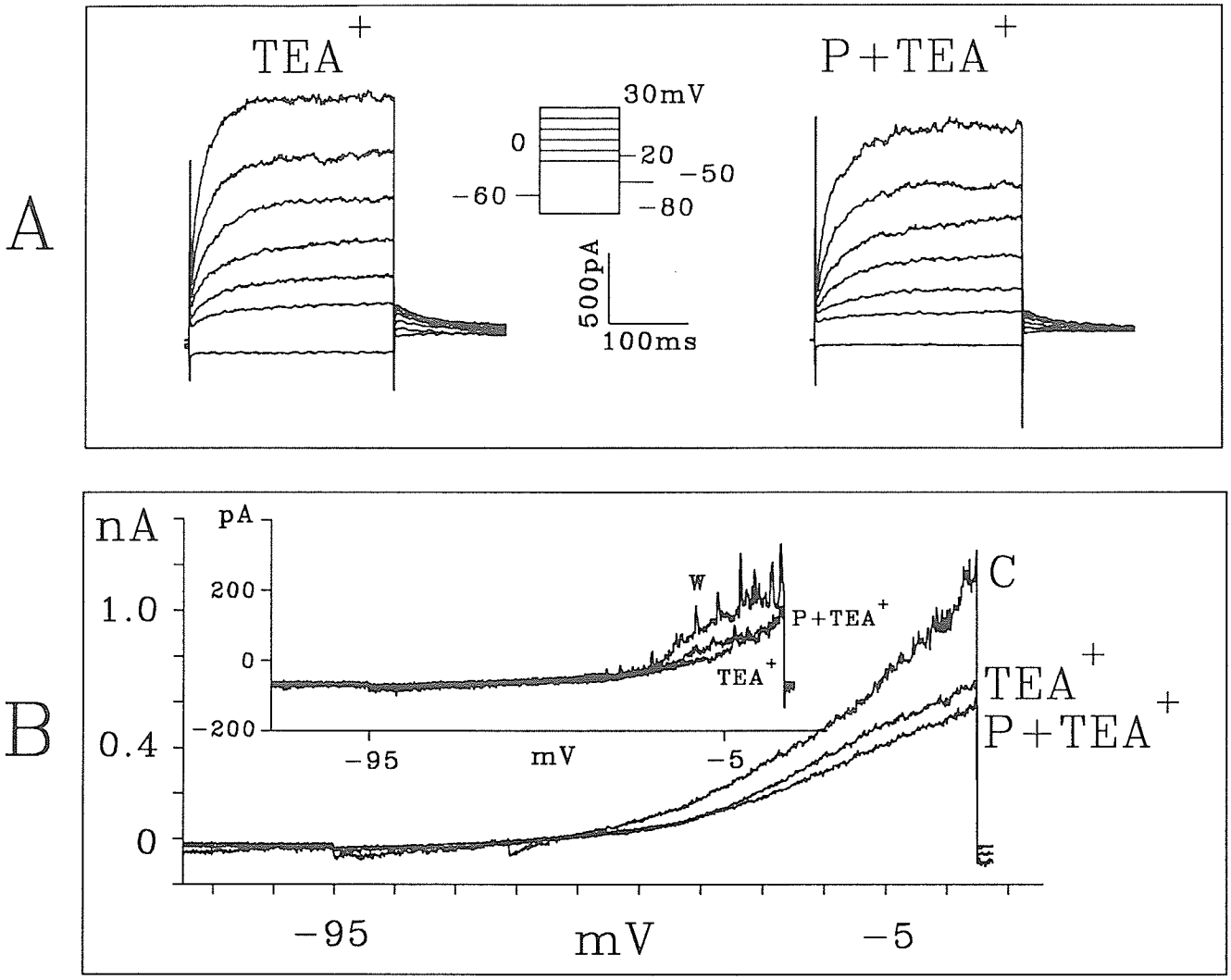

Figure 6: Effect of pinacidil on whole-cell current after pretreatment with TEA^+.

A: Families of membrane currents recorded in the continued presence of 0.5 mM TEA^+ before (TEA^+) and during application of 50 μM pinacidil ($P+TEA^+$) evoked by voltage clamp protocol indicated. ***B:*** Quasi steady-state membrane current evoked by ramp protocol (see legend Fig. 4) in absence (C), presence of 0.5 mM TEA^+ (TEA^+) and during exposure to pinacidil ($P+TEA^+$). Note absences of change in holding current and increase in outward current during combined application of pinacidil and TEA^+. Inset shows similar experiment in which oscillatory outward currents (spontaneous transient outward currents) were apparent after washout of TEA^+ and pinacidil (W).

1986) or oscillatory outward currents (Xiong et al. 1991) that Ca^{2+}-activated K^+ channels were present and functional but blocked by TEA^+ during the pinacidil treatment period (inset, Figure 6B). Figure 7A records the time course of changes in holding current (I_{hold}) and peak steady-state current at +10 mV (I_{ss}) in the presence of pinacidil (50 μM), before and after brief (3 - 6 min) exposure to TEA^+ (0.5 mM), after a second exposure to pinacidil in the presence of TEA^+, and finally upon washout of TEA^+ in the continued presence of pinacidil during a long experiment on one myocyte. Ramp protocols were applied every 45 seconds during the experiment. The corresponding current traces recorded at various times during the experiment are shown in panels B to D of figure 7. Pinacidil induced an outward shift in holding current and increased steady-state outward current during the ramp which were reversed upon washout with control bathing solution (Fig. 7A & B). Application of 0.5 mM TEA^+ to the same cell decreased steady-state outward current (Fig. 7A & C) and in the presence of TEA^+, the magnitude of the shift in holding current and increase in peak steady-state current due to pinacidil were reduced compared to the changes caused by application of the drug alone (Fig. 7A & D). In the presence of TEA^+, the increase in steady-state current at +10 mV due to pinacidil was reduced from 208 to 67 pA, a decline of 68 %, and the increase in holding current was similarly reduced from 15 to 4.4 pA, a decline of 70%. That a similar effect of TEA^+ on the pinacidil sensitive current was evident at +10 mV and holding potential of -70 mV suggests a single conductance contributed to the current at both voltages. When TEA^+ was then removed in the continued presence of pinacidil, holding current and peak steady-state outward current increased, the latter clearly reaching the level observed during the initial exposure to pinacidil and no rundown of the pinacidil-sensitive current during the experiment had therefore occurred.

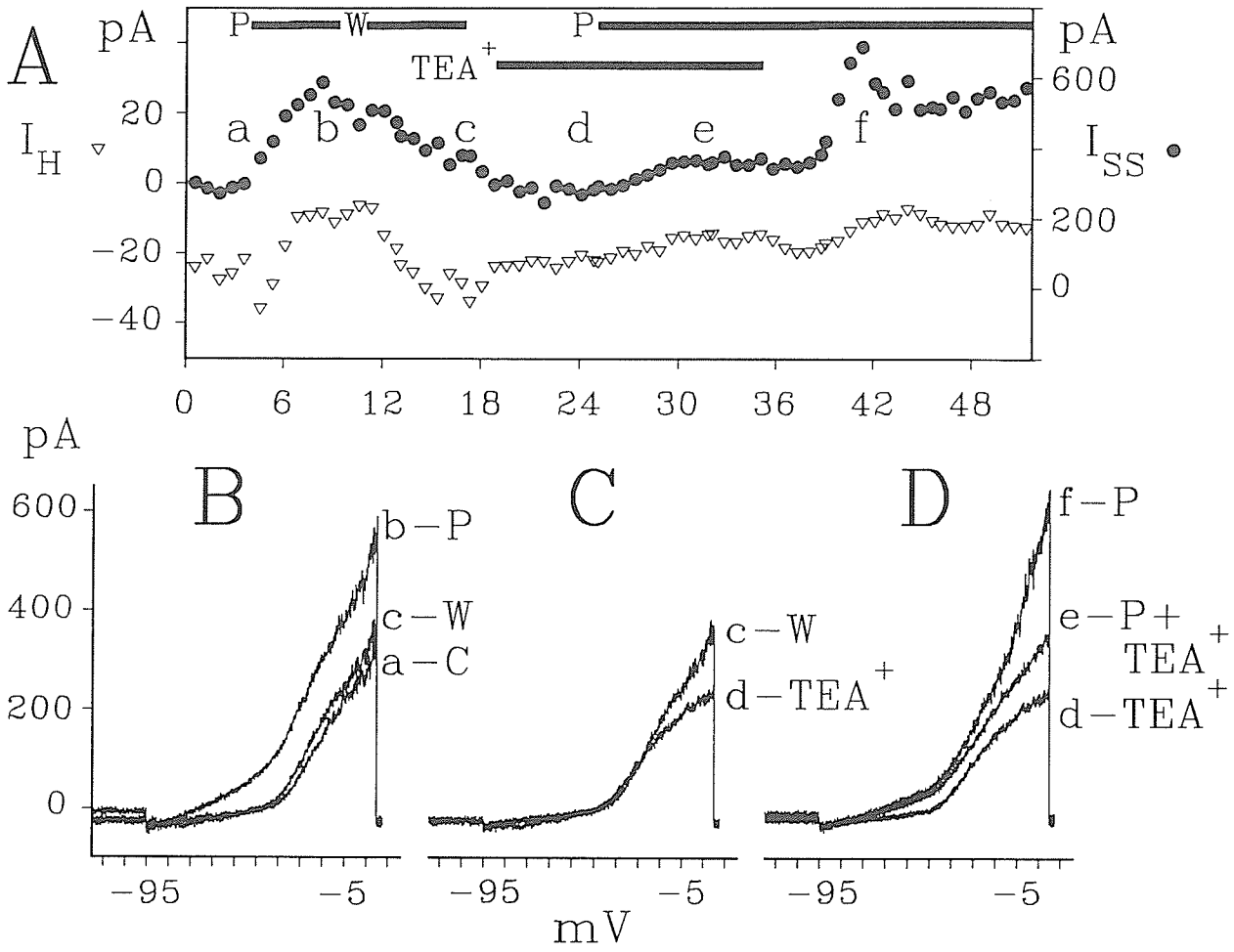

Figure 7: Effect of pinacidil on quasi steady-state whole-cell current after TEA^+.

Current traces recorded from myocyte subsequently exposed to 50 μM pinacidil (P), washout (W), 0.5 mM TEA^+ (TEA^+) and combined exposure to TEA^+ & pinacidil (P + TEA^+) followed by washout of TEA^+ in continued presence of pinacidil (P). ***A:*** Time course for changes in quasi steady-state current at +10 mV (I_{ss} ●) and holding current (I_h ▽). Letters a - f indicate positions during which ramps (see legend Fig. 4) shown in panels B - D were applied during the experiment. ***B-D:*** Current traces (a - f) recorded at times indicated by corresponding letters in panel A.

To further test the possible role of Ca^{2+}-activated K^+ current, the effect of CTX on the pinacidil-enhanced current was studied. 20 nM CTX reversed the enhancement by pinacidil of both holding and peak steady-state current (Fig. 8). Holding current was depressed back to the control level (see inset) and peak current was actually depressed below control levels. Similar results were observed in two other cells.

The data presented above suggest that a Ca^{2+} activated K^+ current could play an important role in the enhancement of whole-cell outward current by pinacidil. However the possibility remained that delayed rectifier K^+ channels may also contribute to the pinacidil-stimulated outward K^+ conductance in these cells, as previously reported with cromakalim (Beech and Bolton 1989b). In light of this the participation of delayed rectifier current in the pinacidil response was investigated. Even in the presence of a high dose of 4-AP (10 mM), exposure to pinacidil resulted in an increase steady-state current, which when measured at the +10 mV level had the same magnitude as was seen with pinacidil in the absence of 4-AP (Fig. 9). Similar results were seen in three other myocytes.

Glibenclamide has been shown to inhibit the actions of several K^+ channel opening drugs in smooth muscle and other tissues (see introduction). For this reason, the influence of glibenclamide on pinacidil-sensitive current was also tested. Figure 10 illustrates that glibenclamide (50 μM) was capable of reversing the changes in outward current induced by 50 μM pinacidil. Similar results were obtained in four other myocytes. Similar to that seen in the presence of CTX, glibenclamide depressed the pinacidil-enhanced current below control levels. This would suggest that glibenclamide is affecting a component of outward current that is not dependent on the presence of pinacidil. This possibility was tested using only glibenclamide. Myocytes were exposed to 50 μM glibenclamide alone (Fig. 11A) which was able to

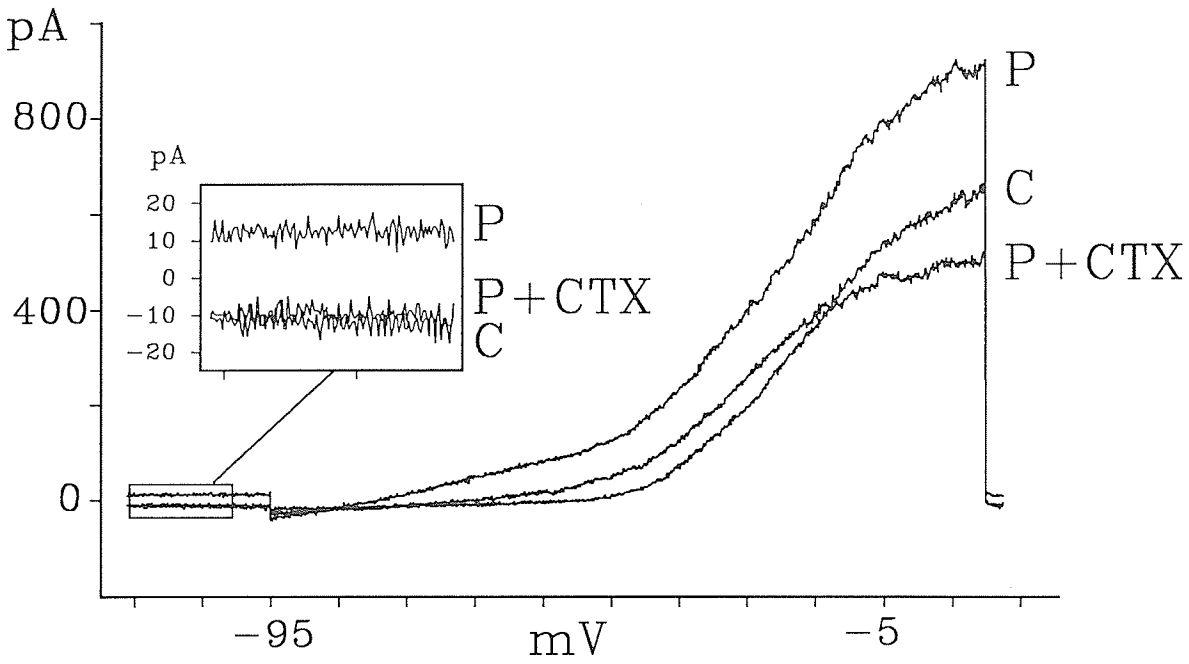

Figure 8: Inhibition of pinacidil-induced steady-state outward current by charybdotoxin.

Quasi steady-state membrane current evoked by ramp protocol (see legend Fig. 4) before (C), after pinacidil (P) and combined pinacidil and charybdotoxin (P + CTX). Note complete reversal of change in holding current (inset) and inhibition of increase in outward current during combined pinacidil and charybdotoxin treatment.

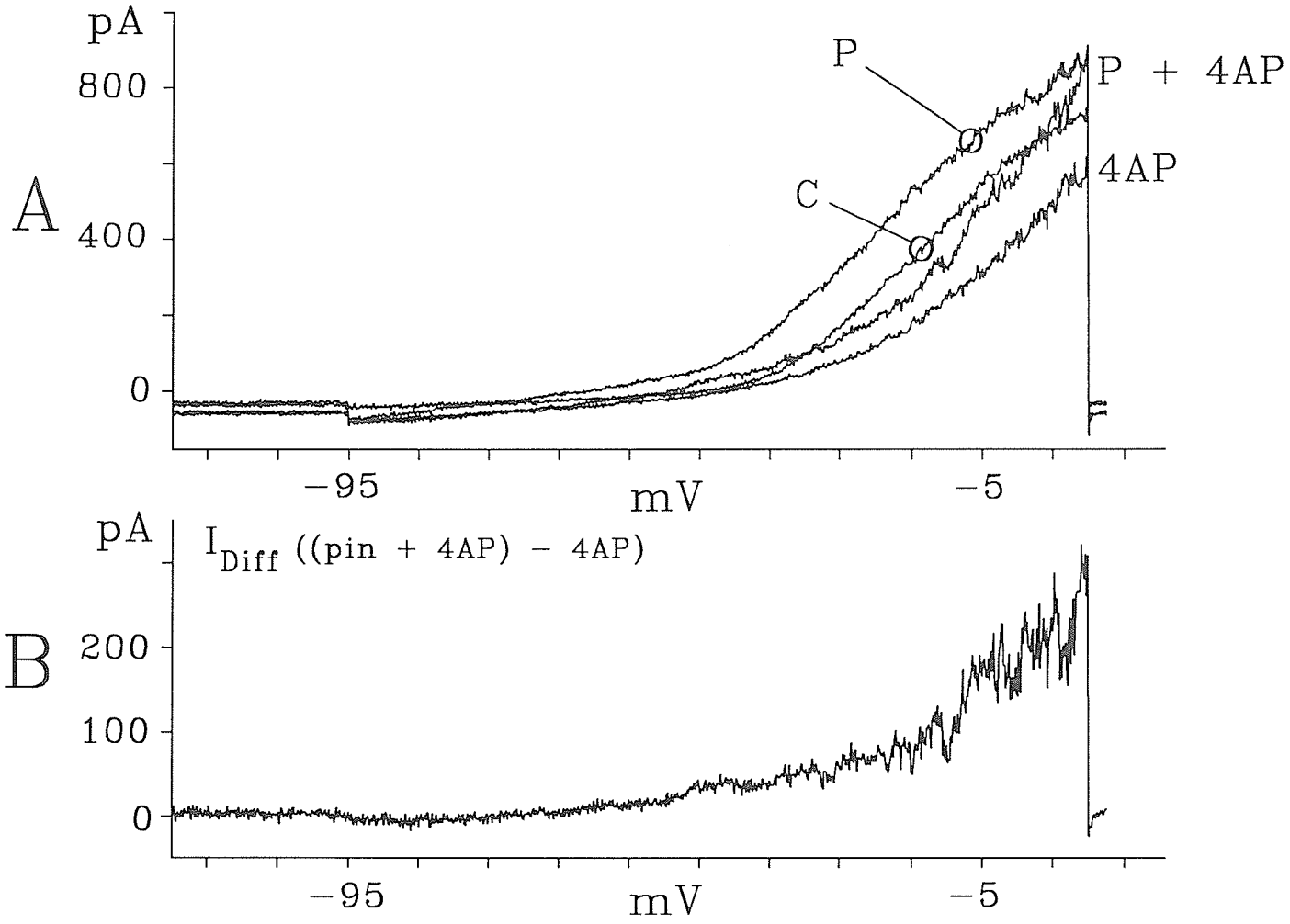

Figure 9: Effect of pinacidil on quasi steady-state whole-cell current in the presence of 4-AP.

Quasi steady-state membrane current evoked by ramp protocol (see legend Fig. 4) before (C) and after addition of 50 μM pinacidil (P). Pinacidil was removed and the current returned to a level comparable with control (W). 4-AP blocks the delayed rectifier portion of the net outward current and reduces overall level of outward current (4-AP). Subsequent addition of pinacidil in the continued presence of 4-AP results in an increased outward current (P + 4-AP) and at the 10 mV level, the outward current has increased to the same degree as in the first exposure to pinacidil.

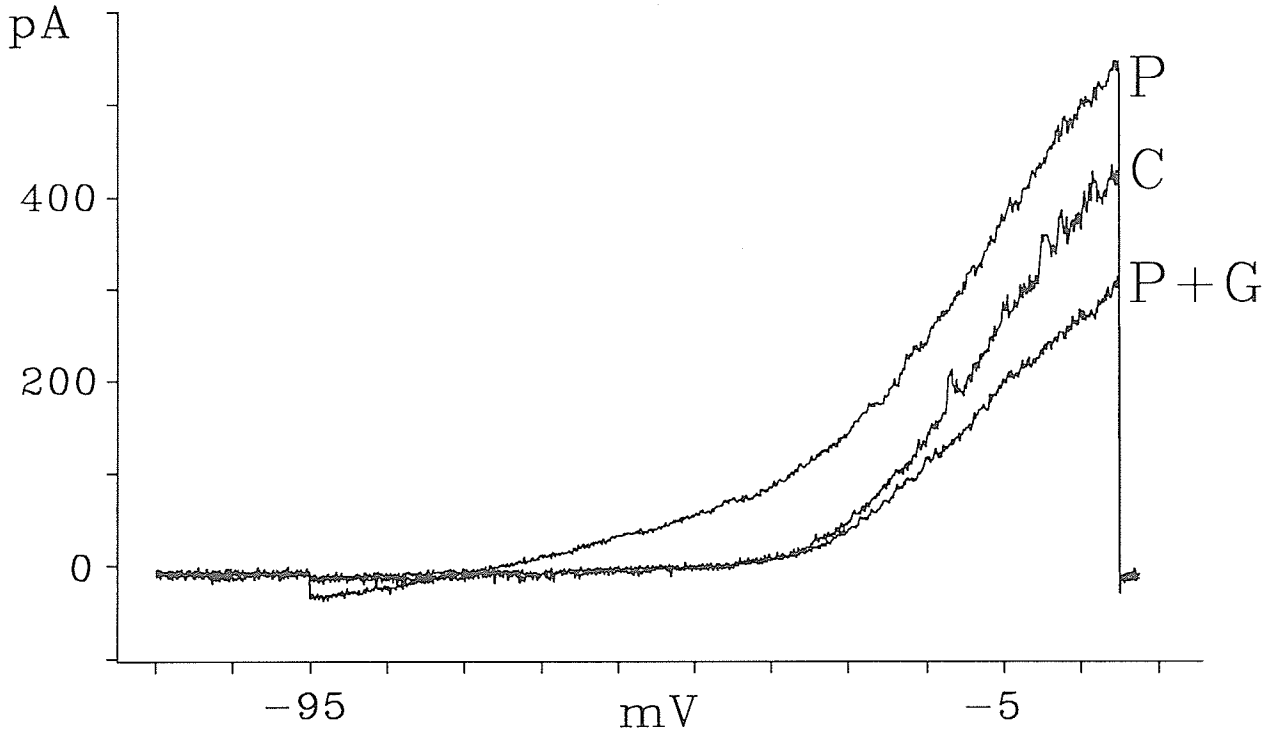

Figure 10: Effect of pinacidil on quasi steady-state whole-cell current in the presence of glibenclamide.

Quasi steady-state membrane current evoked by ramp protocol (see legend Fig. 4) before (C) and after pinacidil (P) and after subsequent treatment with 50 μM glibenclamide (P + G).

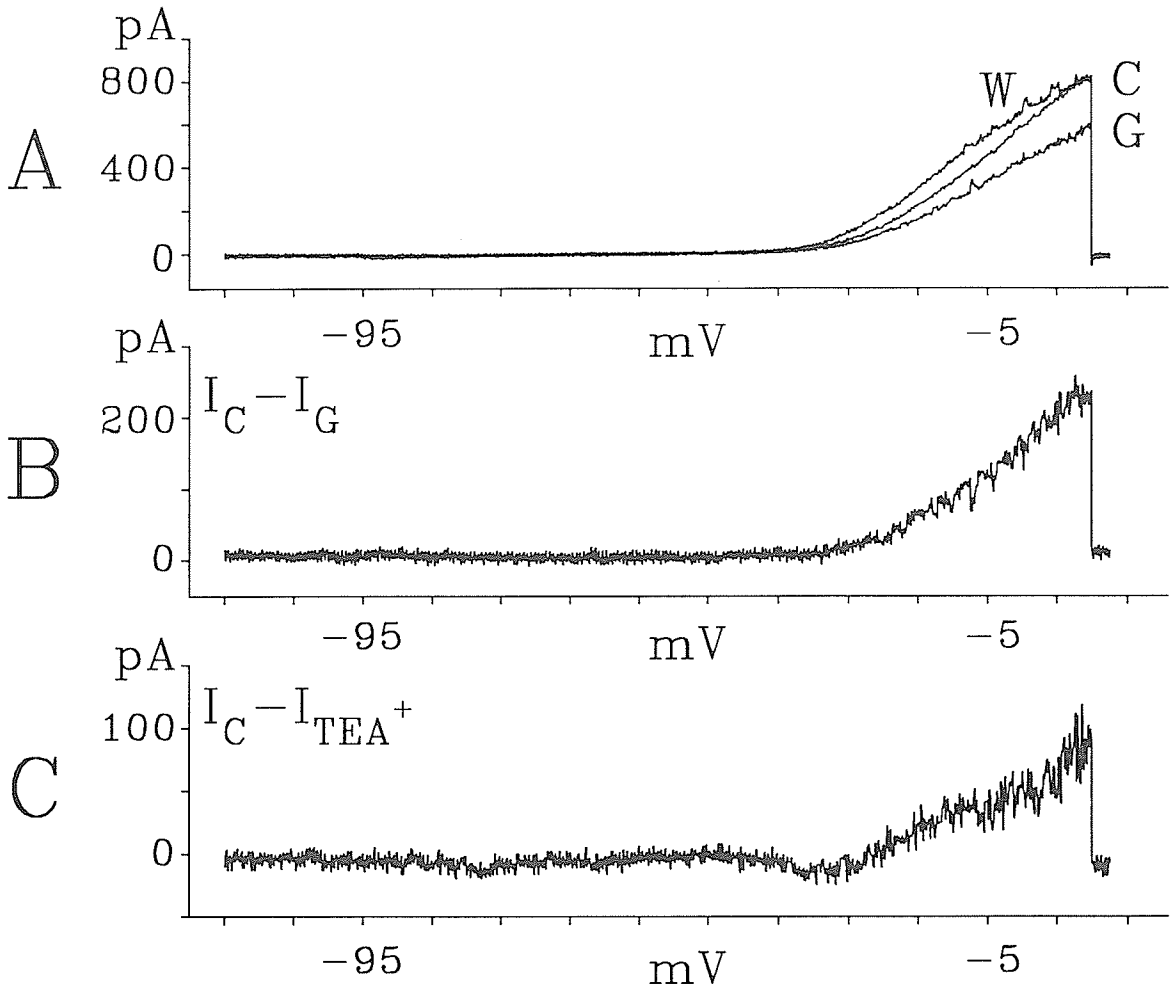

Figure 11: Effect of glibenclamide on quasi steady-state whole-cell current.

Quasi steady-state membrane current evoked by ramp protocol (see legend Fig. 4). ***A:*** Outward currents recorded before (C) and after addition of 50 μM glibenclamide (G) and after washout (W). ***B:*** Difference current obtained by digital subtraction of glibenclamide (G) from control (C) whole-cell current. ***C:*** Similar difference current obtained by digital subtraction of current recorded in the presence of 0.5 mM TEA^+ from control whole-cell current from a myocyte in a separate experiment. Note similarity of voltage-dependence between the glibenclamide and TEA^+ inhibited current.

inhibit the current produced at the positive end of the ramp protocol, compared to control. Digital subtraction of the glibenclamide trace from control yielded a difference current that displayed outward rectification and voltage-dependence (Fig. 11B). Similar results were obtained in four other cells. For comparison, figure 11C also illustrates a difference current obtained by digital subtraction of a current trace recorded in the presence of TEA^+ (0.5 mM) from a trace recorded under control conditions. Similar outward rectification and voltage-dependence can be seen in this example and the striking resemblance of these two difference currents suggests that both these manipulations may influence a similar component of outward K^+ conductance.

Pinacidil causes enhanced activity of a large conductance K^+ channel: Although whole-cell data point to the involvement of a Ca^{2+}-activated K^+ current, more than one Ca^{2+}-activated conductance is known to be present in portal vein myocytes (Inoue et al. 1985). Single channel experiments were therefore conducted to directly identify the K^+ channel(s) stimulated by pinacidil in cell-attached patches. Single channel activity was studied in myocytes bathed in either nominally Ca^{2+}-free, high K^+ solution to set membrane potential to 0 mV or physiological solution in which membrane potential could be altered by pinacidil. As reported elsewhere, three conductances of K^+ channels, approximately 50, 100 and 250 pS, were observed in patches under symmetrical K^+ conditions. Of these, only the 250 pS channels showed a reversible doubling of its activity by pinacidil. Figure 12 illustrates the effects of pinacidil on the activity of a 250 pS channel recorded at a pipette potential of +30 mV (equivalent to a hyperpolarization of -30 mV) before, during and after exposure of the myocyte to pinacidil (50 μM). Downward deflections of current line represent the discrete openings of channels located in the patch and outward current flow through these channels from the cell into the pipette. Given that high K^+ depolarizing

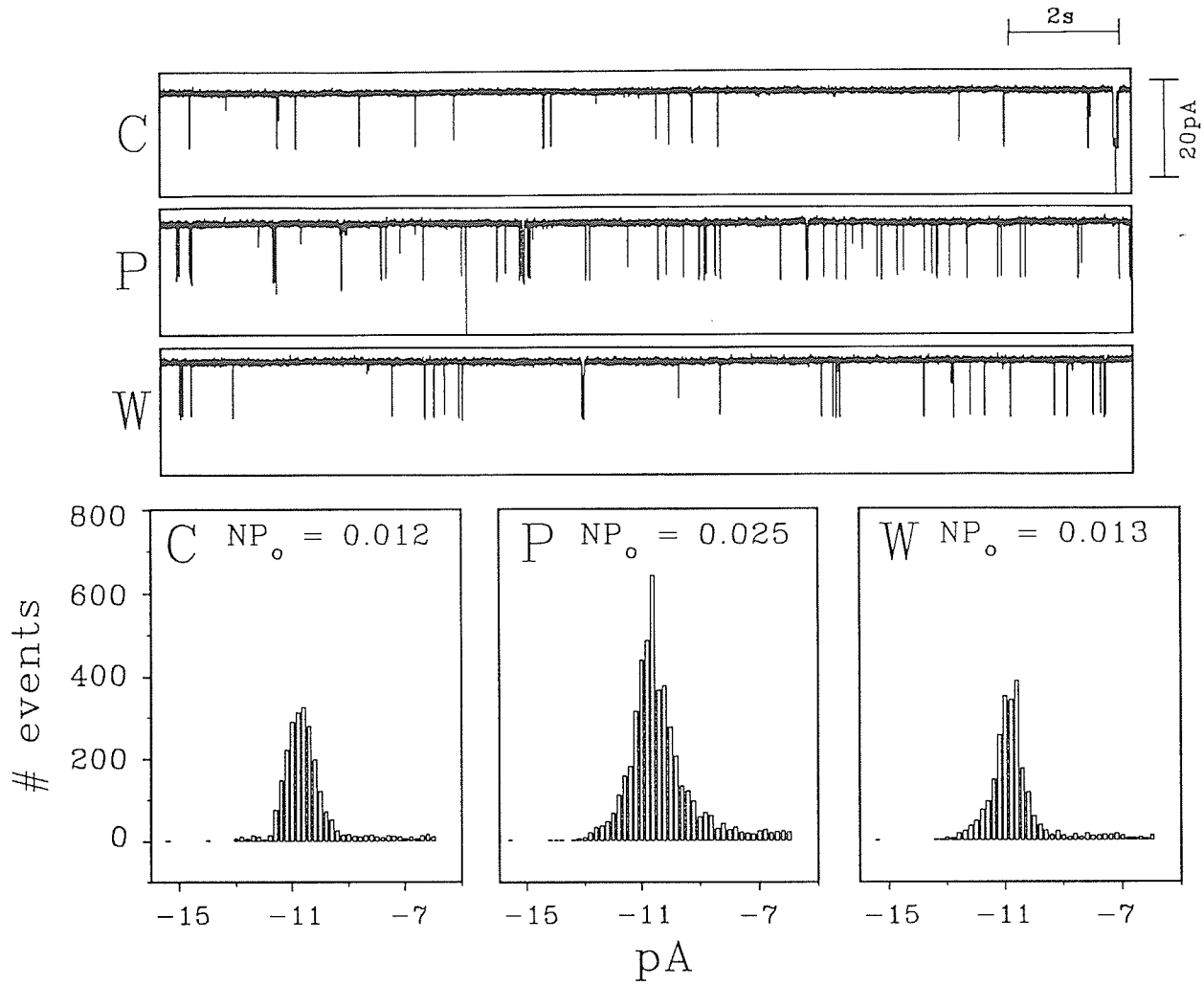

Figure 12: Effect of pinacidil on large conductance channel activity in cell-attached patch.

A: Representative 15 second traces of single channel activity in cell-attached patch from a myocyte bathed in high K^+ solution (symmetrical K^+ in pipette assuming 140 K^+ in cytoplasm) before (C), during (P) and after washout (W) of 50 μM pinacidil at a pipette potential of +30 mV. The unitary currents observed correspond to opening of 250 pS Ca^{2+}-activated K^+ channels. ***B:*** Event histograms for identical 3 minute intervals before (C), during (P) and after washout (W) of pinacidil. Calculated open probability values are given for each period (NP_o = number of channels x mean open probability of single channels; see Methods for additional details).

solution eradicates the cells membrane potential, the electrical driving force is equal to the holding potential, in this case 30 mV. The conductance of these channels can therefore be calculated (g = I/V) giving a value of ~250 pS. Open probability of the large conductance channels, expressed as NP_o (see methods for description and calculation), increased from 0.012 before, to 0.025 during pinacidil and then fell back to 0.013 upon drug washout. A similar increase in large conductance K^+ channel activity due to pinacidil was observed in 6 on-cell patches from cells exposed to high K^+ solution. Additionally similar results were found in 2 patches on cells bathed in normal Krebs (symmetrical K^+ in pipette). Since pinacidil would be expected to induce hyperpolarization, possible changes in channel activity due to depolarization under physiological recording conditions are not thought to have occurred. In two patches (symmetrical K^+), an increase in the activity of a small K^+ channel (~50 pS) was also noted, but the activity of this channel did not appear to decline upon washout of pinacidil.

OBSERVATIONS IN CARDIAC MUSCLE

Pinacidil enhances a voltage-independent steady-state outward K^+ current in cardiac myocytes: In order to compare the effects of pinacidil in smooth muscle to those reported to occur through K_{ATP} channels in cardiac cells, experiments were conducted to show its effect on isolated guinea-pig cardiac myocytes using similar whole-cell recording conditions as described in the experiments on portal myocytes. The effects of 50 μM pinacidil on the action potential and the whole-cell net current recorded from the same cell is shown in figure 13. In the presence of 5 mM EGTA and at 37 but not at 22 °C, pinacidil rapidly reduced the duration of the action potential (Fig. 13A) and caused a large increase in an outward conductance (Fig. 13B). The

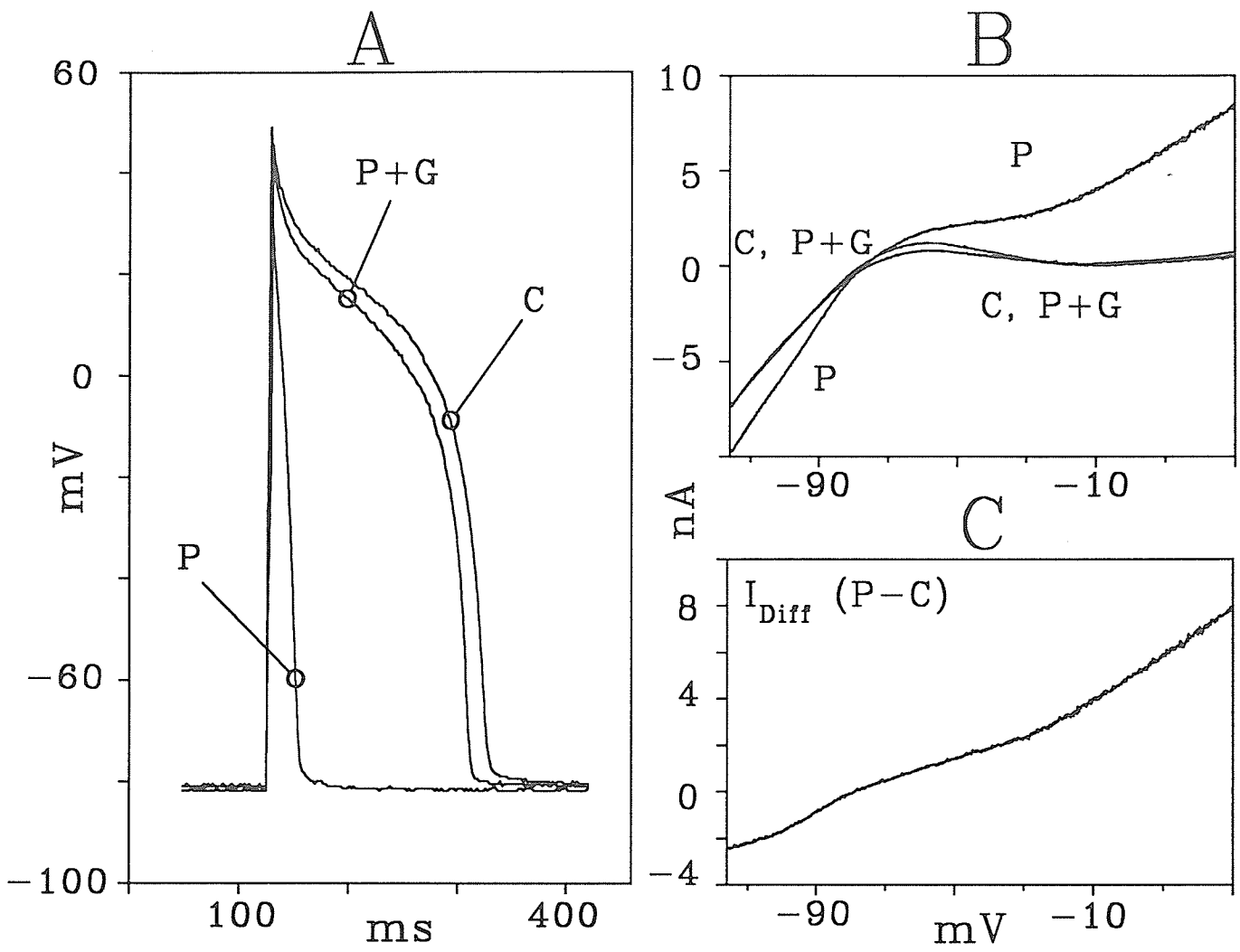

Figure 13: Pinacidil enhances a voltage-independent steady-state outward K^+ current in cardiac myocytes

A: Current-pulse stimulated action potentials and *B:* whole-cell quasi steady-state outward currents evoked by 8 second voltage ramps between -130 and +30 mV from a holding potential of -90 mV recorded from a single isolated guinea-pig ventricular myocyte dialysed with pipette solution containing 5.0 mM EGTA recorded before (C) and during (P) exposed to 50 μM pinacidil and then to combined exposure to pinacidil and 50 μM glibenclamide (P+G). The collapse in the action potential duration occurred within 3 minutes of the addition of pinacidil and these alterations were only seen at 37°C. At room temperature, ~22°C, no effect of pinacidil was observed within 15 min exposure to the drug. *C:* Difference current obtained by subtraction of control trace from that recorded with pinacidil.

effects were seen within 3 minutes exposure at 37 °C (0.5 Hz stimulation) and completely reversed upon exposure to 50 μM glibenclamide. With glibenclamide the action potential duration and quasi-steady state currents rapidly returned back to, but not beyond, the levels recorded under control conditions. In the absence of pinacidil, glibenclamide alone had no effect on the action potential (not shown). The difference current obtained by subtraction of control quasi steady-state current trace from that obtained during the presence of pinacidil (Fig. 13C) reveals that, in cardiac myocytes, the pinacidil-sensitive component of current is voltage-independent over the range of membrane potentials studied.

ALTERATION OF OUTWARD CONDUCTANCE BY *METABOLIC INHIBITION*

OBSERVATIONS IN SMOOTH MUSCLE

Metabolic inhibition stimulates a voltage-, Ca^{2+}- and TEA^+-sensitive outward current: Effects of metabolic inhibition (MI) on quasi steady-state currents in portal vein myocytes were investigated by exposing cells to cyanide (2 mM) and 2-deoxy-D-glucose (10 mM). The addition of cyanide and replacement of glucose by 2-deoxy-D-glucose in the bathing solution resulted in the rapid activation of outward current which reversed close to the calculated E_K value (Fig. 14A). Digital subtraction of control current from MI current (Fig. 14B) clearly demonstrates the voltage-sensitive and outward rectifying nature of the MI-sensitive current. Similar results were achieved in seven other cells. In many instances, after onset of outward current, the myocytes underwent complete contracture and pipette seal was lost ending the experiment. Inclusion of 1 mM TEA^+ in the extracellular solution (Fig. 15) prevented the onset of outward current by cyanide, but replacement of the cyanide + TEA^+

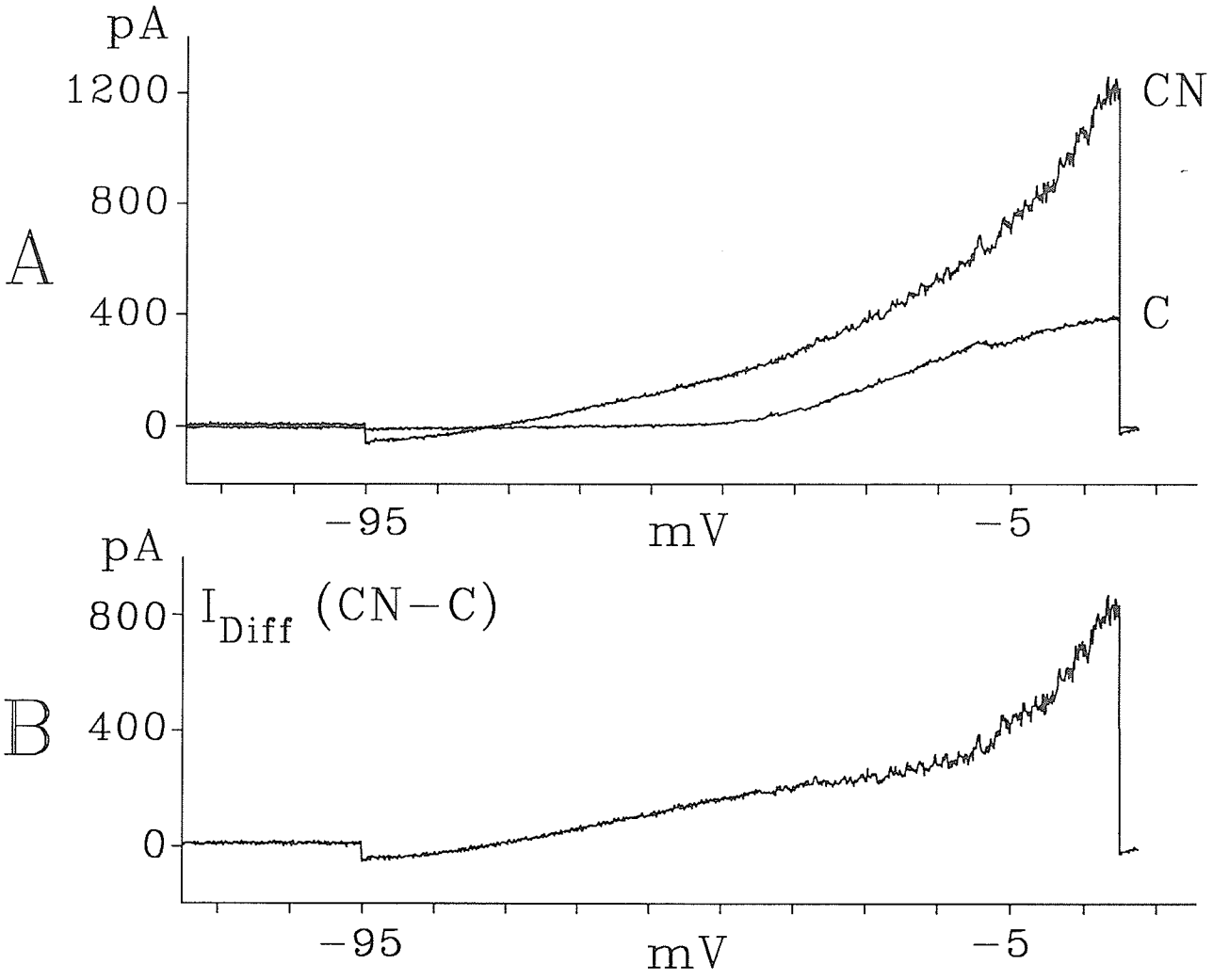

Figure 14: Metabolic inhibition by cyanide and 2-deoxy-D-glucose causes the enhancement of a voltage-dependent outward current in portal vein myocytes.

A: Quasi steady-state outward current recorded from a single isolated portal vein myocyte evoked by ramp protocol (see legend Fig. 4) before (C) and after (CN) the addition of 2 mM cyanide and 10 mM 2-deoxy-D-glucose. ***B:*** Difference current obtained by subtraction of control trace from that recorded during enhancement by MI.

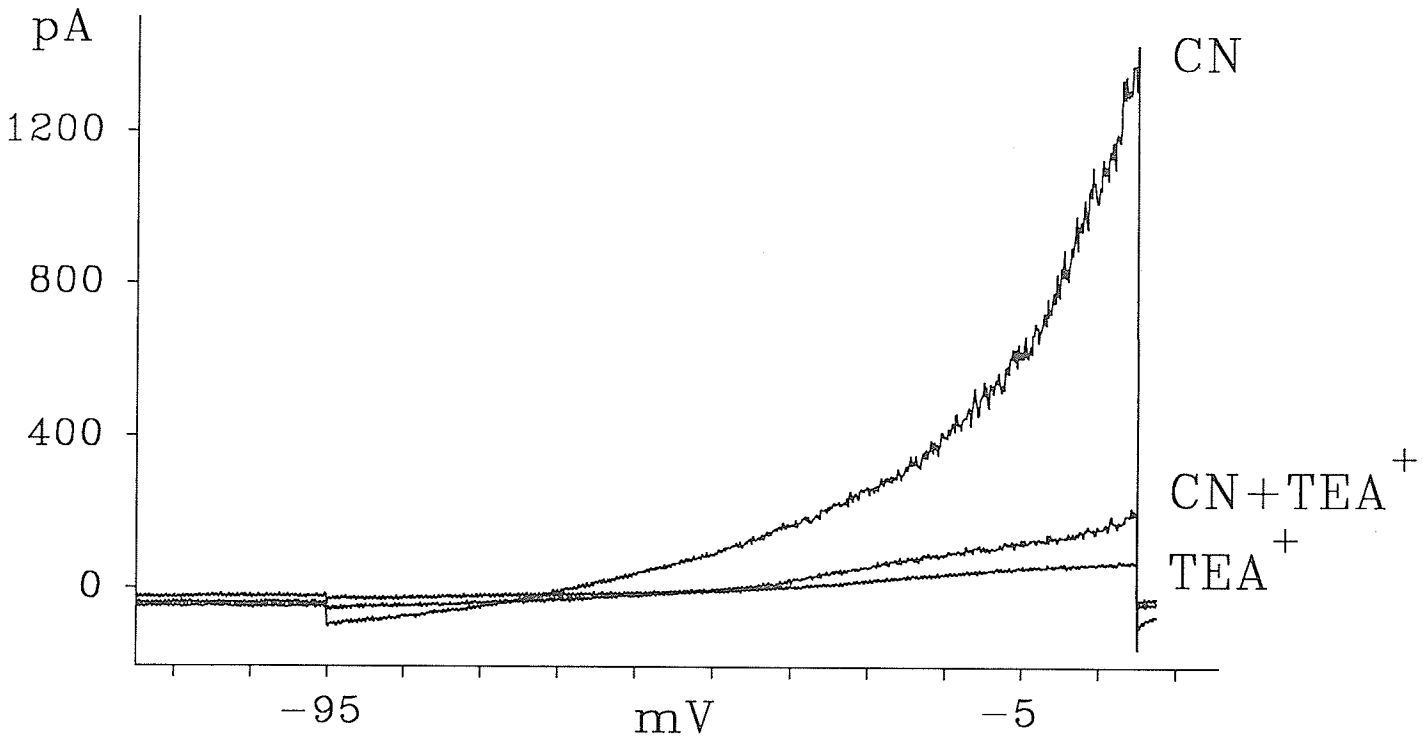

Figure 15: Outward current enhanced by metabolic inhibition is sensitive to low concentrations of TEA^+.

Quasi steady-state outward current recorded by ramp protocol (see legend Fig. 4) from a single isolated portal vein myocyte in the presence of 1 mM TEA^+ (TEA^+), combined presence of TEA^+ and cyanide ($CN + TEA^+$) and then after the removal of TEA^+ from the bathing solution (CN).

solution with one containing only cyanide resulted in a rapid increase in outward current identical to that seen in figure 14. Similar results were seen in three other experiments. In the presence of TEA^+, there was no enhancement of outward current even after 34 minutes of cyanide perfusion. The inclusion of 5 mM EGTA in the pipette solution resulted in a similar prevention of cyanide-induced outward current as was seen with TEA^+ (Fig. 16). The slight decline in current observed after the addition of cyanide with EGTA in the pipette solution may be due to inhibition by cyanide of another outward membrane current or regulating factor, or simply due to rundown of outward K^+ current as previously described to occur in the presence of EGTA (Fig. 5).

Metabolic inhibition causes the enhancement of large conductance K^+ channel activity: The whole-cell data suggests that MI causes the stimulation of a voltage- and Ca^{2+}-sensitive current which is blocked by low concentrations of TEA^+, similar to the component enhanced by pinacidil. It was necessary to carry out single channel studies in order to evaluate the conductance of the channel affected by MI to evaluate whether a similar single channel was affected by both MI or pinacidil. Single channels from on-cell patches were recorded from cells bathed in physiological solutions using symmetrical concentration of K^+ in the pipette. The addition of 2-deoxy-D-glucose with cyanide (Fig. 17) or DNP (Fig. 18) to the bathing solution resulted in a 12 fold increase in the activity of large conductance channels. During single channel recording a series of ramp protocols were executed for two purposes: a) Manipulation of membrane potential before and after cyanide or DNP would ascertain if there was any voltage dependence to the channels affected by MI. b) As these cells were bathed in physiological solutions it was impossible to directly measure resting membrane potential without gaining access to the cell and therefore difficult to establish the

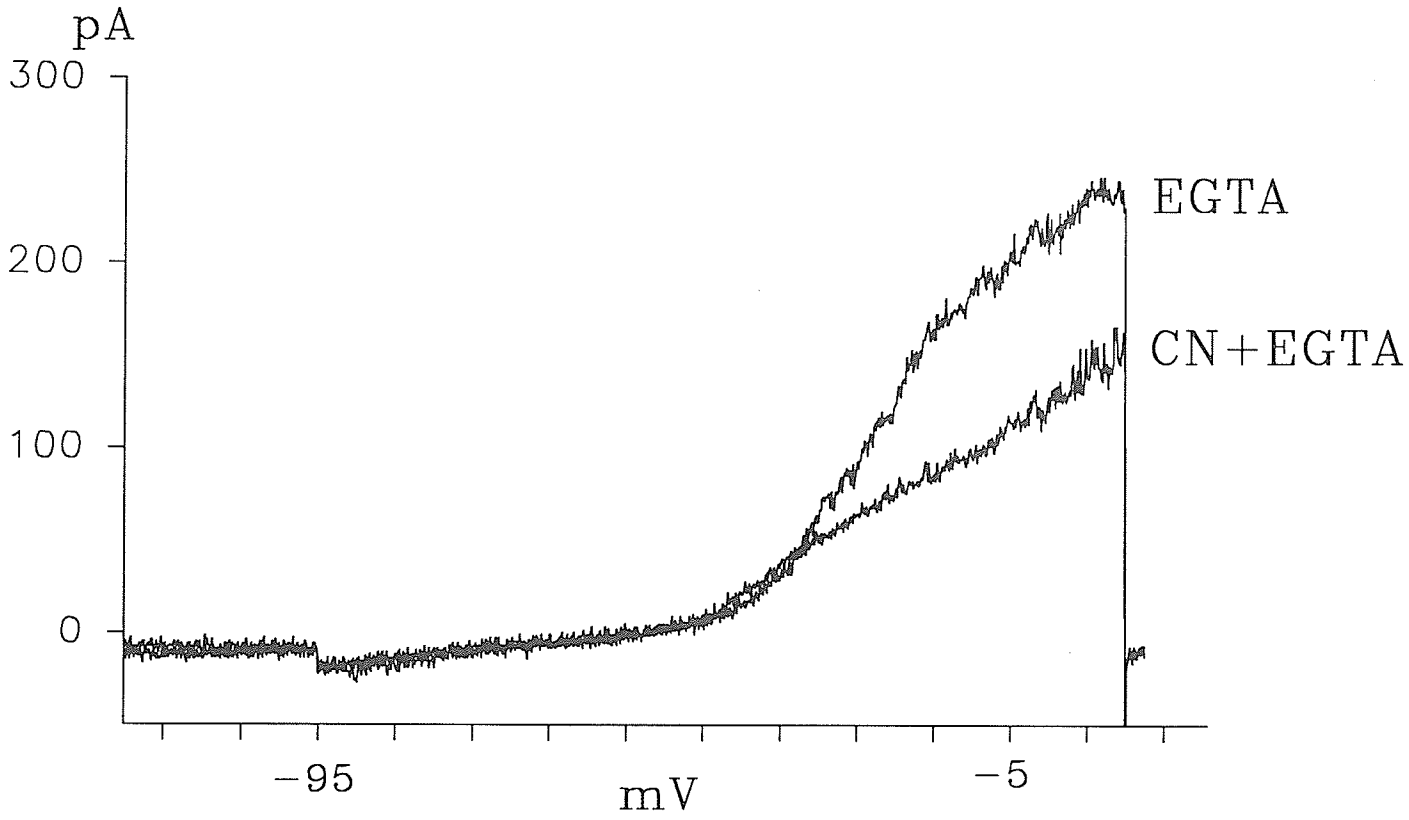

Figure 16: Metabolic inhibition evoked outward current is inhibited by internal application of EGTA

Quasi steady-state outward current recorded by ramp protocol (see legend Fig. 4) from a single isolated portal vein myocyte recorded with 5 mM EGTA in the pipette solution (EGTA) and after 34 minutes of cyanide and 2-deoxy-D-glucose (CN + EGTA) perfusion.

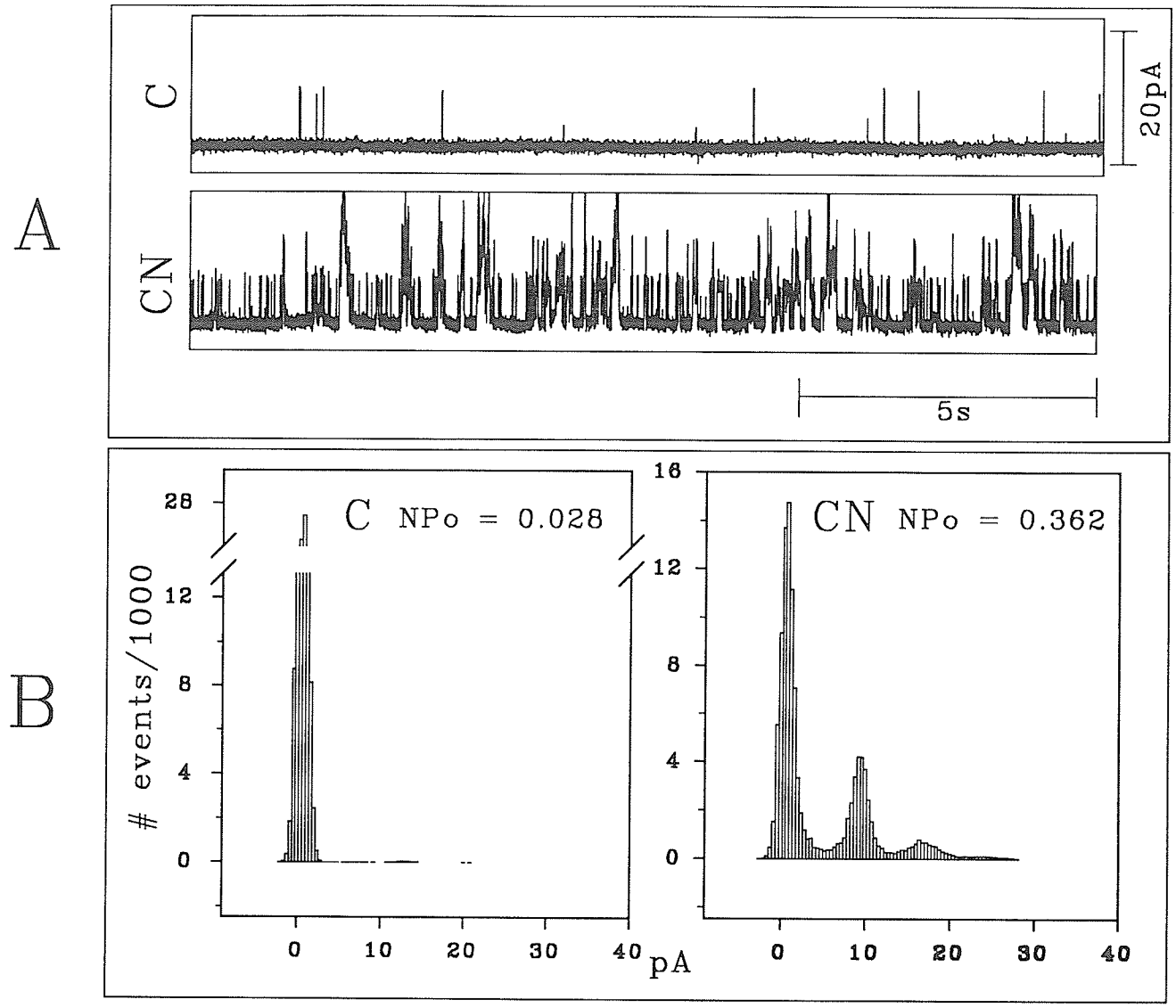

Figure 17: Metabolic inhibition by cyanide enhances the activity of large conductance K^+ channels in portal vein myocytes.

A: Representative 15 second traces of single channel data recorded from a cell bathed in physiological solution under control conditions (C) and later during cyanide (2 mM) and 2-deoxy-D-glucose (10 mM) induced metabolic inhibition (CN). ***B:*** Events histograms calculated for identical durations of 48 seconds before and during metabolic inhibition with corresponding NP_o values. Because more than one channel level was observed the NP_o value was calculated to establish the changes in open probability. Channel conductance was 135 pS (from slope of amplitude change with voltage - see Fig. 20).

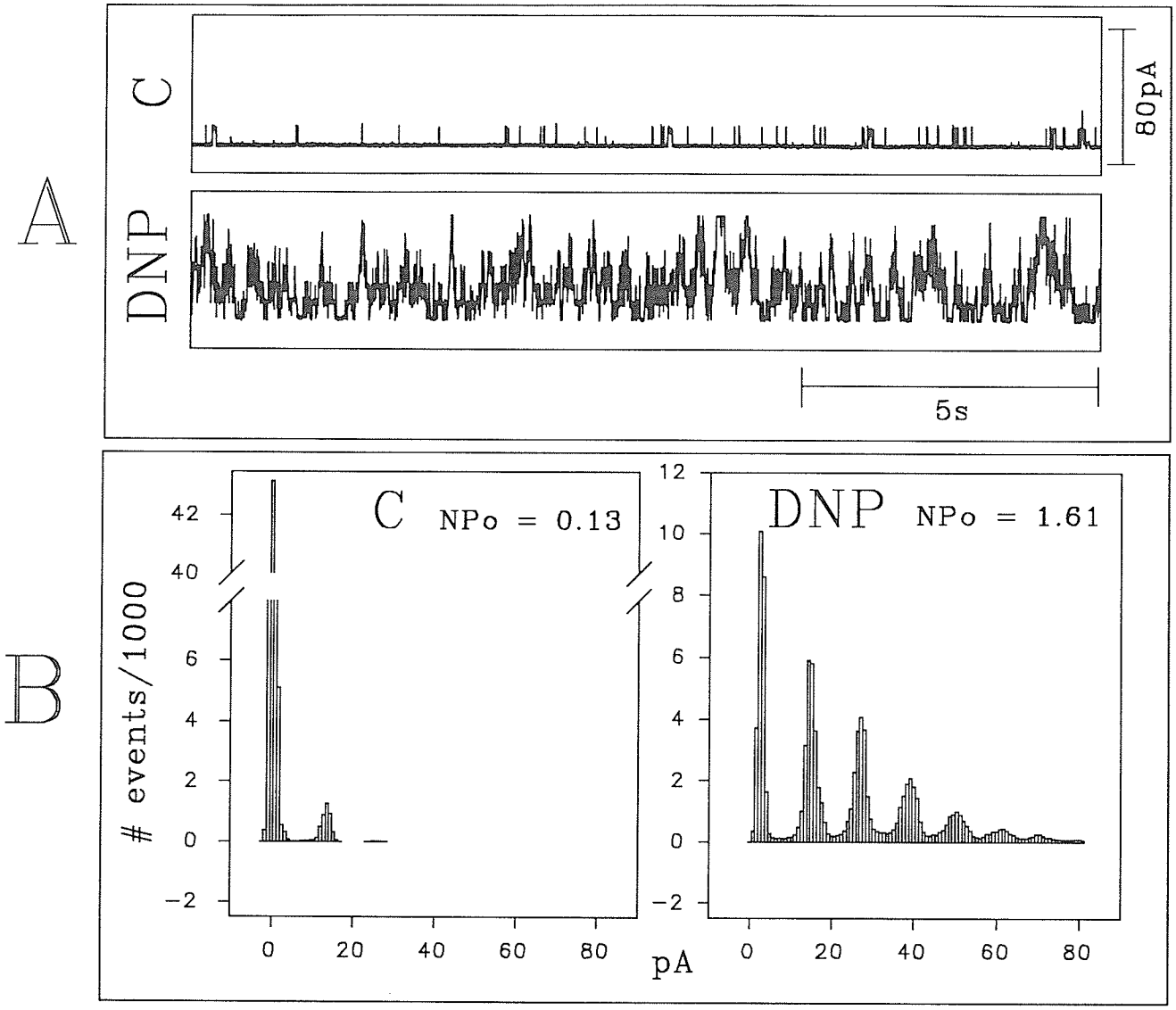

Figure 18: Metabolic inhibition by DNP enhances the activity of large conductance K^+ channels in portal vein myocytes.

A: Representative 15 second traces. (C) control conditions and during DNP (50 μM) and 2-deoxy-D-glucose (10 mM) induced metabolic inhibition (DNP). ***B:*** Events histograms calculated for identical durations of 48 second period before and during metabolic inhibition with corresponding NP_o values. Because there was more than one channel level was observed the NP_o value was calculated to establish the changes in open probability. Channel conductance was 120 pS (calculated from slope of changing amplitude with voltage - see Fig. 20).

electrical driving force needed to calculate channel conductance. For this reason the patch was subjected to ramp protocols before and after the application of cyanide (CN) or DNP (DNP) (Fig. 19). The enhancement of activity of the single channels in both cases clearly occurs to a greater degree at the more negative pipette potentials, which is seen from the perspective of the patch as a depolarization of the transmembranal potential. These channels are therefore undoubtedly voltage dependent. The conductance size of these channels was also determined from these ramp protocols. As is seen in figure 20, the amplitude of the single channel current varied with applied membrane potential, as is evident from the line connecting the peaks of current for one channel level at each voltage. Mathematically, the slope of this line is equivalent to the single channel conductance and was calculated to be approximately 135 and 120 pS in the cyanide and DNP experiments respectively.

OBSERVATIONS IN CARDIAC MUSCLE

Metabolic inhibition stimulates a voltage- and Ca^{2+}-insensitive outward current in isolated cardiac myocytes: As in the pinacidil portion of this study, a comparison between the action of MI in cardiac and smooth muscle cells was done. Effects of MI on action potential and quasi steady-state currents were studied in isolated guinea-pig cardiac myocytes by exposure to cyanide (CN) (2 mM) and 2-deoxy-D-glucose (10 mM). The effect of cyanide and 2-deoxy-D-glucose in a single isolated guinea-pig ventricular myocyte is shown in figure 21. MI resulted in a rapid shortening of the action potential duration (Fig. 21A) as well as concurrent activation of outward current which displayed a reversal potential close to the calculated E_K value in this experiment (Fig. 21B). The difference current produced by digital subtraction of control from MI-induced current (Fig. 21C) displays a linear character (voltage

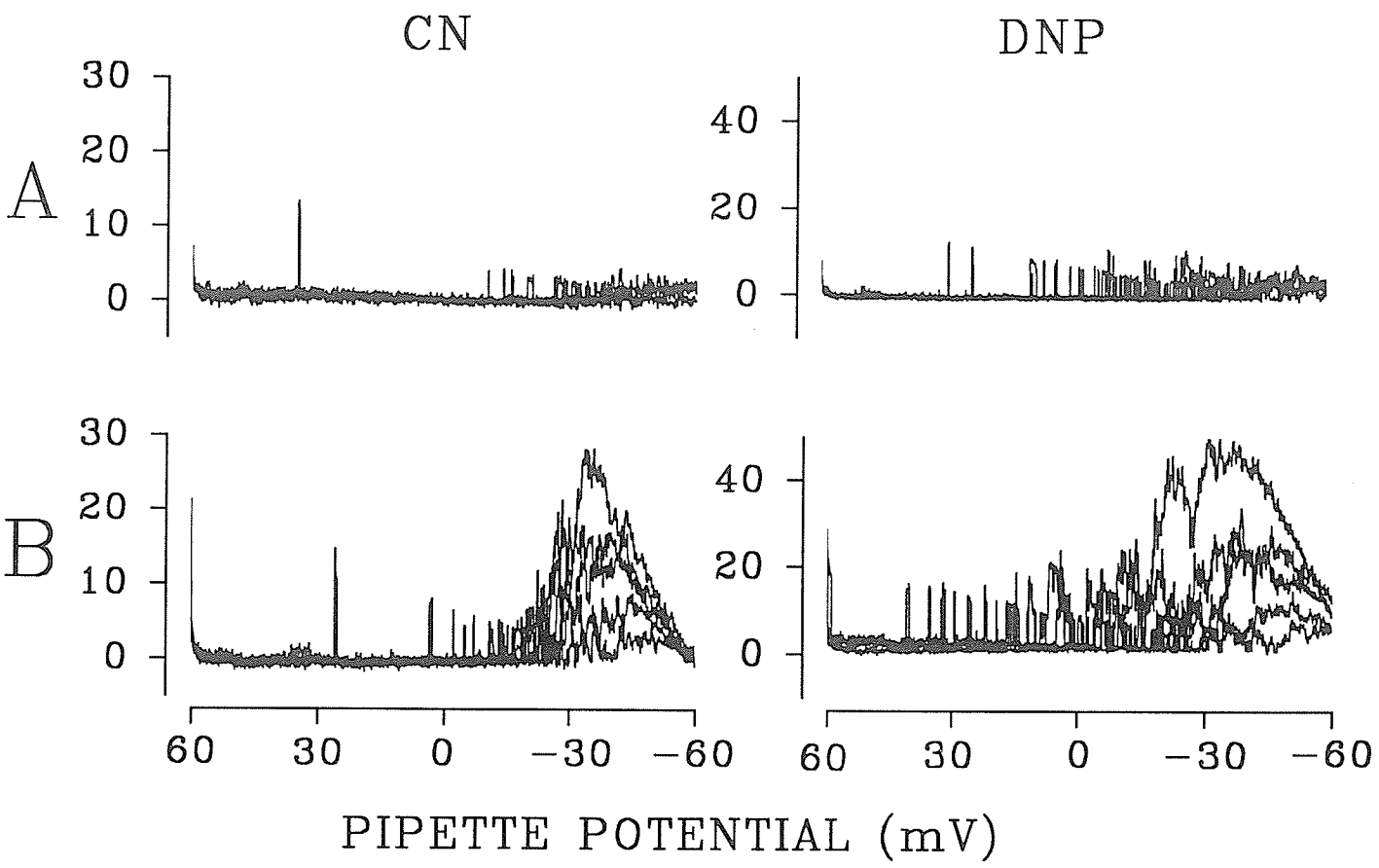

Figure 19: Metabolic inhibition of portal vein myocytes enhances the activation of voltage-dependent large conductance channels.

Single channels events evoked by 4 second voltage ramps between 60 and -60 mV from a holding potential of 0 mV from on-cell membrane patch of a portal vein myocyte bathed in physiological solution and using symmetrical K^+ solution in the pipette. ***A:*** Traces recorded before the addition of 2 mM cyanide (CN) or 50 μM dinitrophenol (DNP) to the bathing solution. ***B:*** Traces recorded from same patches after metabolic poisoning by cyanide (CN) or dinitrophenol (DNP).

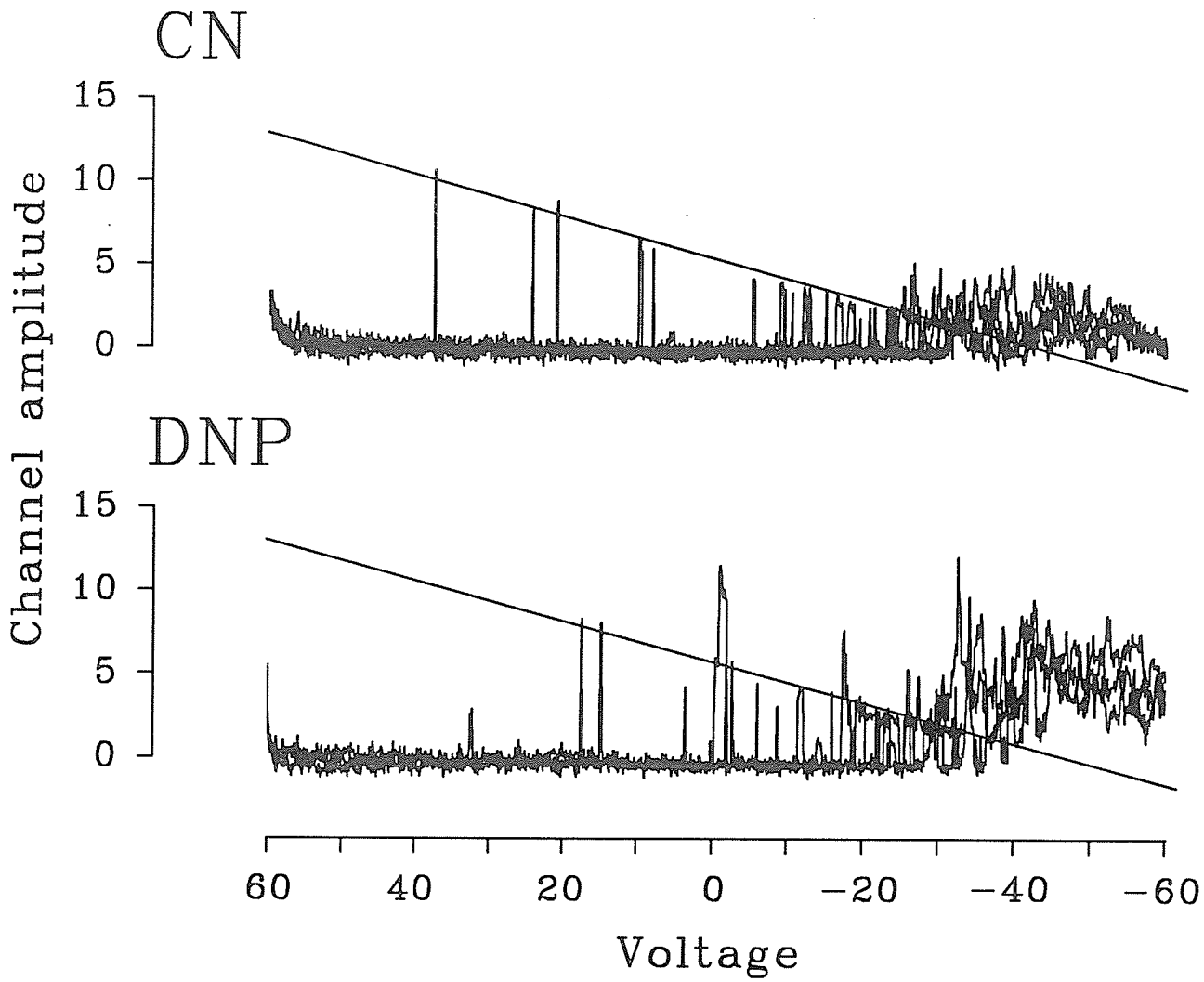

Figure 20: Calculation of channel conductance from patches recorded in cells bathed in physiological solutions.

Single channels events evoked by 4 second voltage ramps between 60 and -60 mV from a holding potential of 0 mV of a portal vein myocyte bathed in physiological solution and using symmetrical K^+ in the pipette solution. These ramps were performed during single channel experiments with cyanide (CN) (Fig. 17) and dinitrophenol (DNP) (Fig. 18) indicate how varying potential (driving force) modifies channel amplitude. Channel conductance is measured from the slope of the line connecting single event amplitudes.

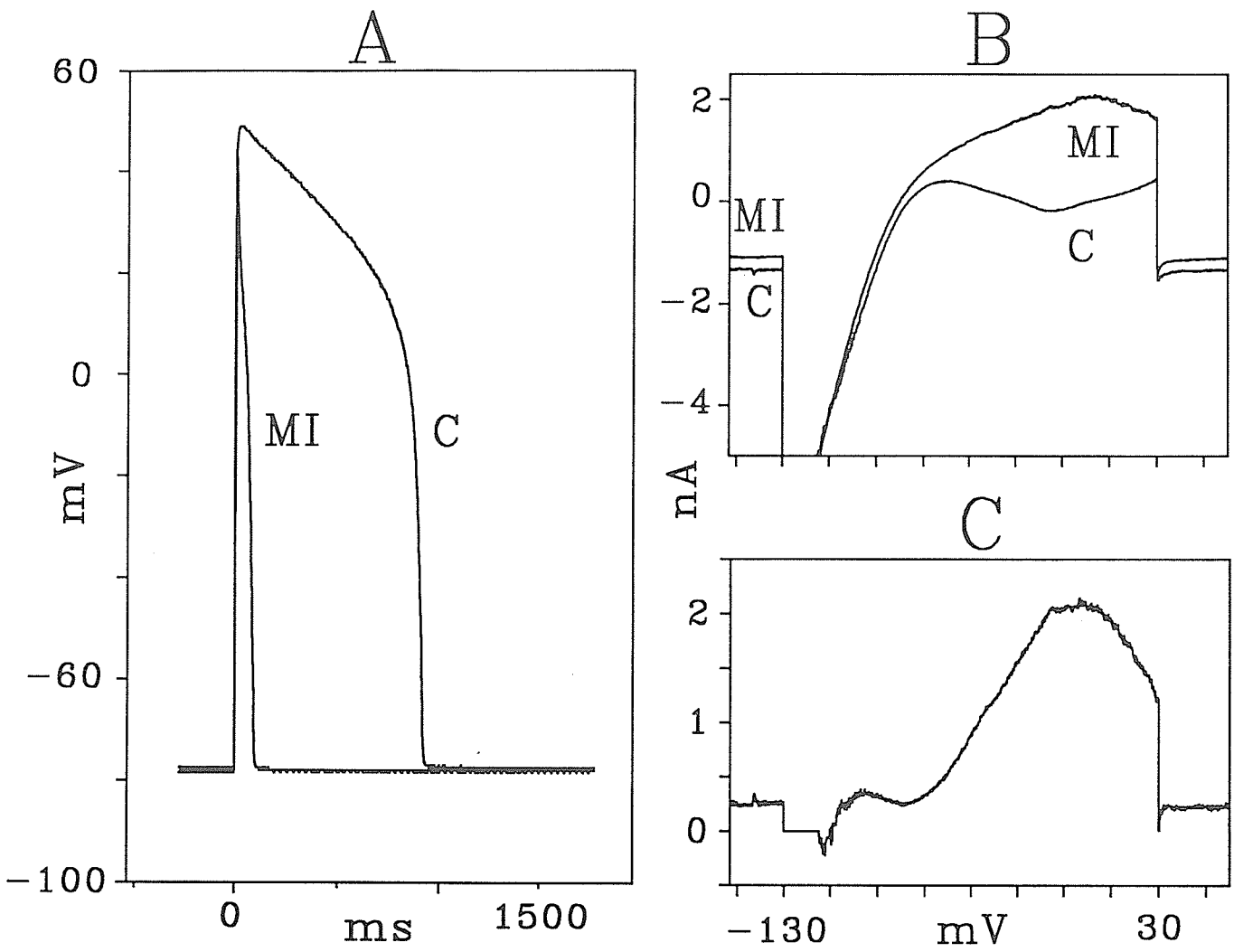

Figure 21: Metabolic inhibition shortens the action potential duration by enhancing a voltage-insensitive outward current in the heart

A: Current-pulse stimulated action potentials and ***B:*** whole-cell quasi steady-state outward currents evoked by 8 second voltage ramps between -130 and +30 mV from a holding potential of -90 mV from a single isolated guinea-pig ventricular myocyte dialysed with pipette solution containing 5.0 mM EGTA and bathed in Krebs, recorded before (C) and after (MI) exposure to 2 mM cyanide. ***C:*** Difference current obtained by subtraction of control trace from that recorded during metabolic inhibition.

insensitive) above -90 mV ($\sim E_K$), and at higher potentials inward rectification is clearly seen in both panels B and C. These characteristics are strong indicators of K_{ATP} current seen in cardiac myocytes (Ashcroft 1988), which has already been reported to be stimulated by MI in these cells (Lederer et al. 1989).

Note in proof: Since the last experiments of this study, further experiments have been conducted in the same laboratory on the effects of metabolic inhibition in smooth muscle cells (pers. comm. Dr E. Morales). When ryanodine was used to unload internal stores of Ca^{2+} prior to experimentation, no effect was observed by the subsequent application of DNP. In other experiments Dr Morales demonstrated the failure of DNP and 2DG to activate any other channel type other than the large conductance K_{Ca} channel when its single channel amplitude was reduced by the presence of 0.1 mM TEA^+, and in excised patches there was no effect of DNP and 2DG on the activity of large K_{Ca}. Other collaborating experiments conducted by Dr N.R. Leblanc using the Ca^{2+} indicator INDO-1 showed that a rise in intracellular Ca^{2+} occurred along with the increases in outward current (see Miller et al. 1993 for a complete description of these experiments and relevant discussion).

DISCUSSION

This study primarily sought to determine the whole-cell outward K^+ current(s) and single channels responsible for the increased membrane permeability to K^+ in isolated smooth muscle cells from the rabbit portal vein in response to either the vasodilating compound, pinacidil, or metabolic inhibition (MI). Whole-cell clamp and cell-attached patch configurations of the patch clamp technique were utilized along with pharmacological agents to permit the identification of the K^+ conductance involved in the alterations of membrane currents by these manipulations.

The results obtained in this study revealed that pinacidil enhances time-dependent and steady-state components of whole-cell current, both exhibiting voltage-dependence and outward rectification. On the basis of intracellular Ca^{2+}-sensitivity, pharmacological and single channel properties, it is concluded that the major outward K^+ current reversibly enhanced by pinacidil in portal vein myocytes is a Ca^{2+}-activated K^+ current carried by large, ~250 pS conductance channels, the so-called maxi-K_{Ca} or BK_{Ca} channel. Stimulation of this current may therefore contribute to the vasodilator actions of pinacidil and its ability to inhibit spontaneous electrical activity in intact preparations. MI also appears to enhance a voltage-dependent outward current, and due to pharmacological and single channel evidence it is believed that this current is also carried through large conductance Ca^{2+}-activated K^+ channels. No strong evidence was found supporting the involvement of any other channel type in this study.

The electrophysiological evidence alone suggests that only one type of channel is involved in the response to pinacidil. At all potentials below 0 mV, application of pinacidil caused an increase in time-dependent outward current, and above 0 mV the

control and pinacidil currents could be seen to intersect (Fig. 3B). This intersection is probably a result of the maximum activation of the outward current at that potential under control conditions, and when fully activated, pinacidil is unable to stimulate it further. Pinacidil induced a leftward shift in the tail current indicating a change in the voltage-dependency of this current. Because there was no change in the slope of the Boltzmann curve it is apparent that pinacidil did not activate another type of channel or cause the recruitment of new channels that were not already present in the sum of control conductances.

The ramp protocols demonstrated pinacidil's ability to enhance steady-state outward current across the range of voltage studied. Of particular interest was the enhancement of outward current between -75 and -45 mV as this illustrates the drug's ability to influence a component of membrane current at a voltage consistent with that of the physiological resting membrane potential. The difference currents calculated for each pinacidil response (not shown) also demonstrated an increase in current noise at more positive voltages. As K_{Ca} is a large conductance channel, the increased current noise is consistent with the involvement of this channel.

The pharmacological evidence also pointed to the involvement of a single channel type. On the basis of only the whole-cell evidence, although it is suggestive, it is impossible to definitely attribute the changes in current across the entire range of ramp potentials to a single channel type. Further experimentation with a variety of pharmacological approaches and single channel measurements were required to identify the conductance(s) affected. Inclusion of EGTA in the pipette solution to chelate free intracellular Ca^{2+} blocked the effect of pinacidil suggesting the involvement of a Ca^{2+}-sensitive conductance supporting previously reported sensitivity of KCO effects to Ca^{2+} (Kreye and Weston 1986, Stockbridge et al. 1991). Since

EGTA could potentially affect any Ca^{2+}-dependent process, including a Ca^{2+}-dependent enzymatic reaction which may stimulate channels not directly sensitive to Ca^{2+}, it was necessary to use additional agents to determine the nature of the conductance. Low concentrations of TEA^+, known to influence the large conductance Ca^{2+}-activated K^+ channel (Inoue et al. 1985), inhibited the ability of pinacidil to enhance outward current. The effects of pinacidil on both time-dependent and steady-state currents demonstrated a parallel sensitivity to intracellular Ca^{2+} chelation and low TEA^+. It is therefore conceivable that both the time-dependent and quasi steady-state currents could in fact be different windows viewing the same conductance. Also, as both holding and peak currents were similarly influenced by pinacidil, and since they were affected to the same degree by low TEA^+, they are likely carried by the same channel. This idea is further supported by the experiments where CTX was used to inhibit K_{Ca} as increases in both holding and peak currents due to pinacidil were blocked by the toxin. In figure 8 note how holding current returned to pre-pinacidil levels after the addition of CTX, but did not fall below control levels suggesting that the CTX-sensitive component, activated by pinacidil at holding potential, is not normally active at that potential without the presence of the KCO. At higher potentials it is noticeable that as well as blocking pinacidil-induced outward current, CTX reduced current to levels considerably below control. This suggests that a CTX-sensitive component which contributes to the outward current under control conditions is only present at these upper potentials. The typical current-voltage relationship for K_{Ca} reported elsewhere (Inoue et al. 1985, Beech and Bolton 1989a, Cole and Sanders 1989) would explain the effect of CTX to only inhibit control current at these higher potentials.

The action of glibenclamide to inhibit the effect of pinacidil in these cells was

remarkably similar to that seen by CTX. Glibenclamide inhibited the pinacidil-induced increase in outward current right across the voltage range as well as reduced peak current to below control levels. Glibenclamide is therefore also able to block a component of outward current available to the cell at these upper potentials under control conditions.

It could be hypothesized that both CTX and glibenclamide influence more than one component of outward current, i.e. pinacidil induced current and another outward current available to the cell but not modulated by pinacidil. The only other K^+ currents present in these cells are the delayed rectifier current, as seen by Beech and Bolton (1989a), and the small conductance Ca^{2+}-activated K^+ channel (Inoue et al. 1985). Delayed rectifier current is not known to be blocked by CTX but is selectively inhibited by 4-AP. Beech and Bolton (1989b) used 4-AP to block the outward current enhanced by cromakalim to conclude that the delayed rectifier was the channel responsible for the KCO response. For this reason 4-AP was included in this study to investigate Beech and Bolton's claim. However in this study, although a very high dose of 4-AP reduced outward current, seen by the ramp protocol, it had no effect on the absolute amount of outward current enhanced by pinacidil. This leads to the conclusion that, at least in this study, delayed rectifier current was not responsible for the increased outward conductance seen by pinacidil.

The single channel investigation also suggests the involvement of a single type of channel conductance. The possibility remained that the smaller conductance Ca^{2+}-sensitive K^+ channel may be affected by pinacidil and so the conductance of the channels which had their activity modified by pinacidil had to be examined. The on-cell patch studies revealed a variety of channel sizes and of these both a large and small channel appeared to be increased in activity after the addition of pinacidil. It is difficult

to rule out other intracellular modulations that may have occurred simultaneously to cause the changes in more than one channel type, and probably not enough control studies were done in this area to see what changes can occur in the control situation over time. However, regardless of this, only the large conductance channel displayed any reversibility to pinacidil, and although the smaller channel became more active, this was probably not a factor influenced by pinacidil, as in the whole-cell and tissue situation, the effects of pinacidil are quite reversible. Although the single channel data here is only suggestive, it is still in general agreement with the major body of evidence created from the whole-cell data which shows that pinacidil is acting through the enhanced activation of large conductance Ca^{2+}-activated K^+ channels.

The data produced from the secondary investigation into the effects of MI on outward membrane currents in portal vein strongly reflected those produced in the pinacidil study. The enhanced outward current was voltage-dependent, Ca^{2+}-sensitive and also inhibited by low (1 mM) concentrations of TEA^+. Here a slightly higher concentration of TEA^+ was used to try to more completely block the outward current assuming that, as the EGTA data suggested, it was K_{Ca} current. Unfortunately a battery of CTX, glibenclamide and 4-AP experiments were not done to investigate the nature of the MI-induced current, but single channel data from both cyanide and DNP experiments supported the findings of the whole cell data.

In these experiments, for the purposes of reproducing physiological conditions, symmetrical solutions were not employed to bath the cells. Using symmetrical K^+ solution only in the pipette, the channels observed had conductances of 135 and 120 pS, smaller than the ~250 pS size recorded in symmetrical K^+ solutions used to bath the cell. The reason for the size discrepancy can be explained if one considers the work of Carl et al. (1990) who observed a drop in the conductance of K_{Ca} channels

recorded in on-cell patches from canine gastric myocytes when the bathing solution was switched from symmetrical to physiological. A similar dependence on extracellular solution in chromaffin cells was noted by Yellen (1984) who observed a change in the conductance of K_{Ca} channels when the extracellular concentration of Na^+ was altered. From this it has been hypothesized that when Na^+ is present at physiological levels in the extracellular solution, increased driving force of Na^+ into the cell keeps $[Na^+]_i$ at a higher value than when the cell is bathed in K^+ symmetrical, nominally Na^+ free solutions. Higher $[Na^+]_i$ may in turn interfere with the free flow of K^+ through the channel and lower its conductance.

In light of these reports and because, when physiological bathing solutions were used, K^+ conductances larger than ~130 pS were not observed it is believed that these channels are the same large conductance channels observed in rabbit portal vein cells when using symmetrical bathing solutions. Furthermore, as clearly demonstrated by the voltage ramps, the channels which had their activity enhanced by MI were voltage dependent, a feature consistent with the idea that they are large conductance Ca^{2+}-activated K^+ channels.

It may be hypothesized that the increase in activity of these channels could occur directly by depolarization or indirectly by an increased Ca^{2+} influx triggered by depolarization. However in most smooth muscle preparations the MI-induced tissue relaxation seen is not consistent with membrane depolarization (Bonnet 1991). The data presented here shows no evidence of a membrane depolarization, indeed although current-clamp measurements were not taken during the application of cyanide of DNP, the shift in the reversal potential of the whole cell current and the reversal for the single channel ramps was not in the positive direction as would be expected with such a membrane depolarization. Although it is not entirely obvious

from the data in figure 19, the reversal point for the recorded current does not appear to have shifted in the positive direction as would be expected by a membrane depolarization. It is therefore believed that the increased activity of these channels was not due to either of these two possibilities.

Many studies have implicated the involvement of channels similar to cardiac K_{ATP} channels, in the KCO-response of smooth muscle (Standen et al. 1989). In order to compare the nature of the response in both cell types, experiments using pinacidil were conducted on isolated guinea-pig cardiac myocytes. The results obtained with pinacidil are identical to those already reported to occur with KCOs in isolated cardiac myocytes (Arena and Kass 1989). Temperature-dependent, Ca^{2+}- and time-independent, inwardly rectifying, and glibenclamide-sensitive current, are all features of K_{ATP} current in this tissue, and are distinctly different in character to those seen in response to pinacidil in rabbit portal vein myocytes. Of particular interest was the fact that the same high EGTA solution, which completely inhibited the effects of pinacidil in smooth muscle cells, was used in these cardiac experiments and yet it had no inhibitory influence on the action of pinacidil. This alone suggests that the channels affected by KCOs in smooth muscle are not identical to the K_{ATP} channels found in the heart.

Further differences between the cardiac K_{ATP} channel and those observed in smooth muscle exist. Standen et al. (1989) conducted their experiments at a similar temperature to that used in the smooth muscle portion of this study, a temperature at which pinacidil was unable to evoke changes in electrical activity in cardiac cells. It is therefore concluded that the mechanism by which the KCOs modulate K^+ channel activity in smooth muscle is different from that in the heart.

Despite the profusion of literature presented in the introduction concerning the

effects of KCOs on smooth muscle contractility, the identity of the K^+ conductance(s) affected by KCOs still remains controversial. This study concludes that pinacidil enhances Ca^{2+}-activated K^+ current in rabbit portal vein myocytes. Such an effect is in agreement with some observations, such as Hu et al. (1990) who observed similar effects to those reported in this study, on a time-dependent outward current and the activity of large conductance Ca^{2+}-activated K^+ channels in rat portal vein with cromakalim and P1060. Lemakalim enhanced a time-independent (steady-state) current in colonic myocytes (Post et al. 1991) by increasing the activity of large conductance Ca^{2+}-activated K^+ channels (Gelband et al. 1991). Both cromakalim and lemakalim reversibly enhanced the activity of large conductance channels in colonic smooth muscle cells, an effect that was sensitive to low (0.2 mM) TEA^+ or glibenclamide (Carl et al. 1992).

However not all the conclusions raised by this study agree with other reported observations. For example, lemakalim was considered to stimulate an outward K^+ current in rat portal vein through small conductance K^+ channels (Noack et al. 1992). Nicorandil activated a low conductance K^+ channel in rat portal vein, but differed from that activated by lemakalim in its demonstrated Ca^{2+}- and ATP-dependence (Kajioka et al. 1990), and cromakalim was concluded to stimulate ATP-sensitive current in rabbit pulmonary arteries from the basis of sensitivity to glibenclamide (Clapp and Gurney 1992). Beech and Bolton (1989b) used rabbit portal vein myocytes, as in this study, but believed that cromakalim enhanced a time-independent current carried by delayed rectifier K^+ channels, based on sensitivity to 4-AP. Several other studies point to effects of KCOs on single channel activities but effects on macroscopic currents have not been well studied in detail. For this reason it is difficult to assess the magnitude of the contribution of these various channels to whole-cell K^+ current.

It is not known why so many diverse conductances are evidently affected by KCOs in smooth muscle, as this is in complete contrast to cardiac muscle where only the ATP-sensitive K^+ channels appear to be activated by the drugs (Arena and Kass 1989, Escande et al. 1989, Fan et al. 1990, Tseng and Hoffman 1990). These discrepancies may be due to a variety of factors such as the source of myocytes, enzymatic isolation conditions used, different recording conditions and/or patch configurations implemented, drug type or concentration, or non specific nature of these drugs for a single channel type which may, even in the same preparation, appear to enhance the activity of multiple kinds of K^+ channels.

Alternatively, it is reasonable to suggest that some of the KCOs affect other cellular processes that modulate K^+ channel activity indirectly. Indeed, it is not at all clear whether KCOs only increase K^+ channel open probability and hyperpolarization. Relaxation in high K^+ solutions by KCOs may be due in part to depression of L-type Ca^{2+} current (Leblanc et al. 1989, Okabe et al. 1990, Nakashima et al. 1990, Post et al. 1991) or modulation of other cell functions such as the blockade of the ryanodine-sensitive outward current in rabbit portal vein (Xiong et al. 1991). This hypothesis, if true, is unlikely to completely account for the action of pinacidil in this study, since an increase rather than a decrease in Ca^{2+}-activated K^+ current was observed. The experiment using high EGTA to inhibit the Ca^{2+}-activated K^+ current may suggest that the action of pinacidil is not direct since pinacidil did not enhance outward current, and therefore pinacidil may require internal Ca^{2+} to act as some kind of second messenger between the receptor site and the channel. Indeed in excised patches, Xiong et al. (1991) reported no modulation of the large conductance K^+ channel by pinacidil, suggesting no direct effect. Nevertheless it could be argued that pinacidil simply requires the presence of Ca^{2+} to function (although this is unlikely as

it is still able to cause K^+ channel activation in cardiac cell with high EGTA), or that the channel itself must in some way be primed by Ca^{2+} before it can respond to the drug.

Glibenclamide has generally been assumed to be a specific antagonist of ATP-sensitive K^+ channels largely based on single channel studies conducted using cardiac myocytes and pancreatic ß-cells (Cook 1988). It has little or no effect on electrical or mechanical responses in healthy cardiac cell or tissue preparations (de Weille and Lazdunski 1990). However, this study indicates that glibenclamide is able to inhibit both (control) current that is available to the cell without the presence of the KCO as well as current activated by pinacidil. Furthermore, as seen in figure 11, the glibenclamide difference current has a similar voltage-dependent I-V relation compared to the low TEA^+ difference current indicating that the channel glibenclamide is inhibiting in the absence of pinacidil is very likely the Ca^{2+}-activated K^+ channel. A similar inhibition of cromakalim-activated time-independent current by 50 μM glibenclamide was previously reported but not attributed to an effect on Ca^{2+}-activated K^+ conductance (Beech and Bolton 1989b). Glibenclamide (1 μM) has been reported to inhibit KCO effects on Ca^{2+}-activated K^+ channels in cell-attached patches (Hu et al. 1990). However, Langton et al (1991) was unable to find any effect of glibenclamide (1 - 100 μM) or cromakalim (5 μM) on Ca^{2+}-activated K^+ channel activity in 8 excised patches of arterial myocytes. This may indicate that glibenclamide does not directly interfere with K_{Ca} channels but actually affects some internal regulating factor such as Ca^{2+} availability from the SR.

However some studies have reported no effect of glibenclamide on the control conductances in smooth muscle cells (Post and Jones 1991), and the reason for this discrepancy is not clear. It is clear, however, that there is little similarity between the

channels activated by KCOs in smooth muscle and K_{ATP} channels seen in the heart or indeed with that seen in pancreas tissue as the concentration of glibenclamide required to block KCO effects in smooth muscle is larger than that needed to block K_{ATP} in either heart or pancreas (Weston and Edwards 1991). Also, as Xiong et al. (1991) have shown, glibenclamide appears to have multiple effects in smooth muscle, and therefore its use as a tool to demonstrate the participation of K_{ATP} channels in various smooth muscle responses, must be questioned. One example of this is a study by Russell et al. (1992) who conducted similar experiments to those presented here, except using lemakalim instead. It was concluded, however, that these effects occurred through the activation of K_{ATP} channels due to the inhibition of the lemakalim-induced effects by glibenclamide. The final conclusion of that study consequently rested on the conviction that glibenclamide was specific for K_{ATP} in that tissue. For that very reason this study did not rely on any one piece of evidence to conclude the activation of large conductance Ca^{2+}-activated K^+ channels and therefore it is believed that this study provides important, strong evidence into the investigation of the nature of the type of K^+ channel modulated by pinacidil.

The ionic basis for resting membrane potential and the contribution of the various types of K^+ channels to electrical activity in rabbit portal vein and other smooth muscles are still poorly defined. This study does not specifically address this issue but it does show that in the presence of pinacidil, Ca^{2+}-activated K^+ current may contribute to resting conductance and shift membrane potential closer to E_K. Although the increase in open probability observed here was small, it is still physiologically significant because in these myocytes with a high input resistance, few large conductance Ca^{2+}-activated K^+ channels need to be active to account for a reasonable change in RMP. Since pinacidil enhanced outward current across the entire

voltage range tested between E_K and +10 mV, it is apparent that the change in conductance may contribute to decreased steady-state and transient Ca^{2+} influx by shifting membrane potential out of the activation range of L-type Ca^{2+} channels and inhibit action potentials. Thus, both resting and developed tone in rabbit portal vein may be expected to decline through effects of pinacidil on the large conductance Ca^{2+}-activated K^+ channels.

It is well established that cardiac myocytes express a time- and voltage-independent current due to ~80 pS conductance ATP-sensitive K^+ channels (Ashcroft 1988) during metabolic depression caused by anoxia (Benndorf et al. 1991), simulated ischemia (Cole 1993), and metabolic poisoning (Lederer et al. 1989). Due to inhibition by glibenclamide of hypoxia-induced vasodilation in coronary arteries it has been concluded that the effects of MI are through the opening of K_{ATP} channels (Duat et al. 1990). Few other studies have been done in this area and the nature of the channels affected in smooth muscle remains unresolved. Because it has been suggested that the channels involved in both the KCO and MI responses of smooth muscle are probably identical due to similar inhibition by glibenclamide (Lydrup and Hellstrand 1991), it seemed useful to investigate this point further. The nature of the currents enhanced by MI seen in this study were almost identical to those seen by the earlier pinacidil data, supporting Lydrup and Hellstrand on the point of identical channels. However the data here did not point to the involvement of the K_{ATP} channel, as the whole-cell characteristics were somewhat different, resembling that of the large conductance Ca^{2+}-activated K^+ channel. A similar comparison was made between the effects of MI in rabbit portal vein myocytes and cardiac cells of the guinea-pig. MI stimulated a Ca^{2+}-independent, steady-state, inwardly rectifying outward current, suggesting the involvement of K_{ATP} current known to be activated by MI in this tissue

(Noma 1983, Lederer et al. 1989). Again these characteristics are quite unlike those displayed by the action of MI in portal vein myocytes. It is therefore concluded that the main conductance responsible for the large increase in outward K^+ current by MI in portal vein myocytes is due to the activation of these large conductance Ca^{2+}-activated K^+ channels, similar if not identical to those seen to be affected by the application of KCOs in these cells, and not ATP-sensitive K^+ channels like those seen in the heart.

It is not conclusive from this study whether the enhanced activation of K_{Ca} is due to a direct affect of the poisons, or through an indirect mechanism, such as a second messenger, or increase in internal Ca^{2+}. However the continued investigation done by Dr E. Morales have demonstrated that the action of DNP and 2DG on single channel activity is not a direct effect, as MI had no influence on the activity of channels recorded in the excised patch. Also the lack of an effect on channel activity after the pretreatment of ryanodine (which causes slow release of intracellular Ca^{2+} stores), suggests that the massive increase in large conductance Ca^{2+}-activated K^+ channel activity observed in cell-attached patches may be due to dumping of intracellular Ca^{2+} stores which may also account for the rise in measured $[Ca^{2+}]_i$ levels observed by Dr Leblanc. This is in contrast to the earlier report of Lederer et al. (1989) where minimal influence on the internal Ca^{2+} stores of cardiac cells by MI was observed, demonstrating additional differences between cardiac and smooth muscle in their responses to MI. The lack of modulation of any other channel other than the large conductance K^+ channel shown by the TEA^+ experiments suggest that only that channel is involved. These additional experiments support the conclusions raised by this study that, in portal vein myocytes, the single channels activated by complete metabolic inhibition are large conductance Ca^{2+}-activated K^+ channels.

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